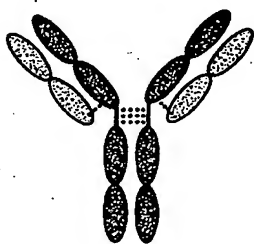




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(54) Title: USE OF BI-SPECIFIC ANTIBODIES FOR PRE-TARGETING DIAGNOSIS AND THERAPY



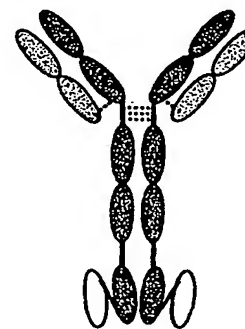
hMN14



734scFv



hMN14Fab-734scFv



hMN14-734scFv

(57) Abstract

The present invention relates to a bi-specific antibody or antibody fragment having at least one arm that specifically binds a targeted tissue and at least one other arm that specifically binds a targetable conjugate. The targetable conjugate comprises a carrier portion which comprises or bears at least one epitope recognizable by at least one arm of said bi-specific antibody or antibody fragment. The targetable conjugate further comprises one or more therapeutic or diagnostic agents or enzymes. The invention provides constructs and methods for producing the bi-specific antibodies or antibody fragments, as well as methods for using them.

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USE OF BI-SPECIFIC ANTIBODIES FOR PRE-TARGETING DIAGNOSIS AND THERAPY

10 Background of the Invention

Field of the Invention

The invention relates to immunological reagents for therapeutic use, for example, in radioimmunotherapy (RAIT), and diagnostic use, for example, in radioimmunodiagnosis (RAID) and magnetic resonance imaging (MRI). In particular, the invention relates to bi-specific antibodies (bsAb) and bi-specific antibody fragments (bsFab) which have at least one arm that specifically binds a targeted tissue and at least one other arm that specifically binds a targetable conjugate. Further, the invention relates to monoclonal antibodies that have been raised against specific immunogens, humanized and chimeric monoclonal bi-specific antibodies and antibody fragments having at least one arm that specifically binds a targeted tissue and at least one other arm that specifically binds a targetable conjugate, DNAs that encode such antibodies and antibody fragments, and vectors for expressing the DNAs. Earlier provisional patent applications, U.S.S.N. 60/090,142 and U.S.S.N. 60/104,156 disclose a part of what is now included in this invention and are incorporated herein by reference in their entireties.

Related Art

An approach to cancer therapy and diagnosis involves directing antibodies or antibody fragments to disease tissues, wherein the antibody or antibody fragment can target a diagnostic agent or therapeutic agent to the disease site. One approach to this methodology which has been under investigation, involves the use of bi-specific monoclonal antibodies (bsAb) having at least one arm that specifically binds a targeted diseased tissue and at least one other arm that specifically binds a low molecular weight hapten. In

this methodology, a bsAb is administered and allowed to localize to target, and to clear normal tissue. Some time later, a radiolabeled low molecular weight hapten is given, which being recognized by the second specificity of the bsAb, also localizes to the original target.

5 Although low MW haptens used in combination with bsAbs possess a large number of specific imaging and therapy uses, it is impractical to prepare individual bsAbs for each possible application. Further, the application of a bsAb/low MW hapten system has to contend with several other issues. First, the arm of the bsAb that binds to the low MW hapten must bind with high
10 affinity, since a low MW hapten is designed to clear the living system rapidly, when not bound by bsAb. Second, the non-bsAb-bound low MW hapten actually needs to clear the living system rapidly to avoid non-target tissue uptake and retention. Third, the detection and/or therapy agent must remain associated with the low MW hapten throughout its application within the bsAb protocol
15 employed.

 Of interest with this approach are bsAbs that direct chelators and metal chelate complexes to cancers using Abs of appropriate dual specificity. The chelators and metal chelate complexes used are often radioactive, using radionuclides such as cobalt-57 (Goodwin *et al.*, U.S. Patent No. 4,863,713),
20 indium-111 (Barbet *et al.*, U.S. Patent No. 5,256,395 and U.S. Patent No. 5,274,076, Goodwin *et al.*, *J. Nucl. Med.*, 33:1366-1372 (1992), and Kranenborg *et al.*, *Cancer Res* (suppl.), 55:5864s-5867s (1995) and *Cancer* (suppl.) 80:2390-2397 (1997)) and gallium-68 (Boden *et al.*, *Bioconjugate Chem.*, 6:373-379, (1995) and Schuhmacher *et al.*, *Cancer Res.*, 55:115-123
25 (1995)) for radioimmuno-imaging. Because the Abs were raised against the chelators and metal chelate complexes, they have remarkable specificity for the complex against which they were originally raised. Indeed, the bsAbs of Boden *et al.* have specificity for single enantiomers of enantiomeric mixtures of chelators and metal-chelate complexes. This great specificity has proven to be a
30 disadvantage in one respect, in that other nuclides such as yttrium-90 and bismuth-213 useful for radioimmunotherapy (RAIT), and gadolinium useful for MRI, cannot be readily substituted into available reagents for alternative uses. As a result iodine-131, a non-metal, has been adopted for RAIT purposes by

using an I-131-labeled indium-metal-chelate complex in the second targeting step. A second disadvantage to this methodology requires that antibodies be raised against every agent desired for diagnostic or therapeutic use.

Thus, there is a continuing need for an immunological agent which can
5 be directed to diseased tissue and can specifically bind to a subsequently administered targetable diagnostic or therapeutic conjugate.

Objects of the Invention

It is one object of the present invention to provide a bi-specific antibody
10 or antibody fragment having at least one arm that specifically binds a targeted tissue and at least one other arm that specifically binds a targetable conjugate that can be modified for use in a wide variety of diagnostic and therapeutic applications.

Other objects of the invention are to provide pre-targeting methods of
15 diagnosis and therapy using the combination of bi-specific antibody and targetable conjugate, methods of making the bi-specifics, and kits for use in such methods.

In accomplishing the foregoing object, the present inventors have discovered that it is advantageous to raise bsAbs against a targetable conjugate
20 that is capable of carrying one or more diagnostic or therapeutic agents. By utilizing this technique, the characteristics of the chelator, metal chelate complex, therapeutic agent or diagnostic agent can be varied to accommodate differing applications, without raising new bsAbs for each new application. Further, by using this approach, two or more distinct chelators, metal chelate
25 complexes or therapeutic agents can be used with the inventive bsAb.

Summary of the Invention

The present invention relates to a bi-specific antibody or antibody
fragment having at least one arm that specifically binds a targeted tissue and at
30 least one other arm that specifically binds a targetable conjugate.

In one embodiment, the invention provides a method of treating or identifying diseased tissues in a patient, comprising:

- (A) administering to the patient a bi-specific antibody or antibody fragment having at least one arm that specifically binds a targeted tissue and at least one other arm that specifically binds a targetable conjugate;
- (B) optionally, administering to the patient a clearing composition, and allowing the composition to clear non-localized antibodies or antibody fragments from circulation;
- (C) administering to the patient a first targetable conjugate which comprises a carrier portion which comprises or bears at least one epitope recognizable by the at least one other arm of the bi-specific antibody or antibody fragment, and one or more conjugated therapeutic or diagnostic agents, or enzymes; and
- (D) when the targetable conjugate comprises an enzyme, further administering to the patient
- 1) a prodrug, when the enzyme is capable of converting the prodrug to a drug at the target site; or
 - 2) a drug which is capable of being detoxified in the patient to form an intermediate of lower toxicity, when the enzyme is capable of reconvertng the detoxified intermediate to a toxic form, and, therefore, of increasing the toxicity of the drug at the target site, or
 - 3) a prodrug which is activated in the patient through natural processes and is subject to detoxification by conversion to an intermediate of lower toxicity, when the enzyme is capable of reconvertng the detoxified intermediate to a toxic form, and, therefore, of increasing the toxicity of the drug at the target site, or
 - 4) a second targetable conjugate which comprises a carrier portion which comprises or bears at least one epitope recognizable by the at least one other arm of the bi-specific antibody or antibody fragment, and a prodrug, when the enzyme is capable of converting the prodrug to a drug at the target site.

In another embodiment, the invention provides a kit useful for treating or identifying diseased tissues in a patient comprising:

(A) a bi-specific antibody or antibody fragment having at least one arm that specifically binds a targeted tissue and at least one other arm that specifically binds a targetable conjugate;

(B) a first targetable conjugate which comprises a carrier portion which comprises or bears at least one epitope recognizable by the at least one other arm of the bi-specific antibody or antibody fragment, and one or more conjugated therapeutic or diagnostic agents, or enzymes; and

(C) optionally, a clearing composition useful for clearing non-localized antibodies and antibody fragments; and

(D) optionally, when the first targetable conjugate comprises an enzyme,

1) a prodrug, when the enzyme is capable of converting the prodrug to a drug at the target site; or

2) a drug which is capable of being detoxified in the patient to form an intermediate of lower toxicity, when the enzyme is capable of reconvertng the detoxified intermediate to a toxic form, and, therefore, of increasing the toxicity of the drug at the target site, or

3) a prodrug which is activated in the patient through natural processes and is subject to detoxification by conversion to an intermediate of lower toxicity, when the enzyme is capable of reconvertng the detoxified intermediate to a toxic form, and, therefore, of increasing the toxicity of the drug at the target site, or

4) a second targetable conjugate which comprises a carrier portion which comprises or bears at least one epitope recognizable by the at least one other arm of the bi-specific antibody or antibody fragment, and a prodrug, when the enzyme is capable of converting the prodrug to a drug at the target site.

Another embodiment of the invention is to provide DNA constructs which encode such antibodies or antibody fragments. Specifically, DNA constructs which produce the variable regions which provide the advantageous properties of reactivity to a targetable conjugate and reactivity to a disease tissue. In accordance with this aspect of the present invention, there is provided a recombinant DNA construct comprising an expression cassette capable of producing in a host cell a bi-specific antibody or antibody fragment having at least one arm that specifically binds a targeted tissue and at least one other arm that specifically binds a targetable conjugate, wherein the construct comprises, in the 5' to 3' direction of transcription, a transcriptional initiation regulatory region functional in the host cell, a translational initiation regulatory region functional in the host cell, a DNA sequence encoding the bi-specific antibody or antibody fragment, and a transcriptional and translational termination regulatory region functional in the host cell, wherein the bi-specific antibody or antibody fragment is under the control of the regulatory regions.

Another embodiment of the invention provides a method of preparing the antibodies or antibody fragments by recombinant technology. In accordance with this aspect of the present invention, there is provided a method of preparing a bi-specific antibody or antibody fragment having at least one arm that specifically binds a targeted tissue and at least one other arm that specifically binds a targetable conjugate, comprising:

- (A) introducing the recombinant DNA construct described above into a host cell;
- (B) growing the cell and isolating the antibody or antibody fragment.

In another embodiment of the present invention there is provided a method of preparing a bi-specific fusion protein having at least one arm that specifically binds to a targeted tissue and at least one other arm that is specifically binds to a targetable conjugate, comprising:

- (1) (A) introducing into a host cell a recombinant DNA construct comprising an expression cassette capable of producing in the host cell a fragment of the bi-specific fusion protein, wherein the construct comprises, in the 5' to 3' direction of transcription, a

transcriptional initiation regulatory region functional in the host cell, a translational initiation regulatory region functional in the host cell, a DNA sequence encoding a scFv linked to a light-chain antibody fragment, and a transcriptional and translational termination regulatory region functional in the host cell, wherein the fragment of the bi-specific fusion protein is under the control of the regulatory regions;

(B) co-introducing into the host cell a recombinant DNA construct comprising an expression cassette capable of producing in the host cell a Fd fragment which is complementary to the light-chain antibody fragment in (A) and which when associated with the light-chain antibody fragment forms a Fab fragment whose binding site is specific for the targeted tissue, wherein the construct comprises, in the 5' to 3' direction of transcription, a transcriptional initiation regulatory region functional in the host cell, a translational initiation regulatory region functional in the host cell, a DNA sequence encoding a Fd fragment, and a transcriptional and translational termination regulatory region functional in the host cell, wherein the Fd fragment is under the control of the regulatory regions;

(C) growing the cell and isolating the bi-specific fusion protein, or

(2) (A) introducing into a first host cell a recombinant DNA construct comprising an expression cassette capable of producing in the first host cell a fragment of the bi-specific fusion protein, wherein the construct comprises, in the 5' to 3' direction of transcription, a transcriptional initiation regulatory region functional in the first host cell, a translational initiation regulatory region functional in the first host cell, a DNA sequence encoding a scFv linked to a light-chain antibody fragment, and a transcriptional and translational termination regulatory region functional in the first host cell, wherein the fragment of the bi-

specific fusion protein is under the control of the regulatory regions;

- 5 (B) introducing into a second host cell a recombinant DNA construct comprising an expression cassette capable of producing in the second host cell a Fd fragment which is complementary to the light-chain antibody fragment in (2)(A) and which when associated with the light-chain antibody fragment forms a Fab fragment whose binding site is specific for the targeted tissue, wherein the construct comprises, in the 5' to 3' direction of transcription, a transcriptional initiation regulatory region functional in the second host cell, a translational initiation regulatory region functional in the second host cell, a DNA sequence encoding a Fd fragment, and a transcriptional and translational termination regulatory region functional in the second host cell, wherein the Fd fragment is under the control of the regulatory regions;
- 10 (C) growing the first and second host cells;
- (D) optionally isolating the bi-specific fusion protein fragment and the Fd fragment; and
- 15 (E) combining the fragments to produce a bi-specific fusion protein and isolating the bi-specific fusion protein.
- 20

A variety of host cells can be used to prepare bi-specific antibodies or antibody fragments, including, but not limited to, mammalian cells, insect cells, plant cells and bacterial cells. In one embodiment, the method utilizes a mammalian zygote, and the introduction of the recombinant DNA construct produces a transgenic animal capable of producing a bi-specific antibody or antibody fragment.

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A further embodiment of the invention involves using the inventive antibody or antibody fragment in photodynamic therapy.

30 A further embodiment of the invention involves using the inventive antibody or antibody fragment in radioimmunoimaging for positron-emission tomography (PET).

A further embodiment of the invention involves using the inventive antibody or antibody fragment in radioimmunoimaging for single photon emission.

- 5 A further embodiment of the invention involves using the inventive antibody or antibody fragment in magnetic resonance imaging (MRI).

A further embodiment of the invention involves using the inventive antibody or antibody fragment in boron neutron capture therapy (BNCT).

- 10 Additional aspects, features and advantages of the invention will be set forth in the description which follows, and in part will be obvious from the description, or may be learned by practice of the invention. The embodiments and advantages of the invention may be realized and obtained by means of the instrumentalities and combinations particularly pointed out in the appended claims.

Brief Description of the Drawings

- 15 Figure 1 schematically illustrates various Abs and bsAbs.

Figure 2 provides SDS-PAGE analysis of purified hMN14Fab-734scFv.

Figure 3 schematically illustrates two bi-specific fusion proteins.

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Figure 4 illustrates the production of a DNA construct useful for producing a hMN14Fab-734scFv bi-specific fusion protein.

- 25 Figure 5 illustrates the production of a DNA construct useful for producing a hMN14Fab-734scFv bi-specific fusion protein.

Detailed Description of Preferred Embodiments

- 30 The present invention provides a bi-specific antibody or antibody fragment having at least one arm that specifically binds a targeted tissue and at least one other arm that specifically binds a targetable conjugate. The targetable conjugate comprises a carrier portion which comprises or bears at least one epitope recognized by at least one arm of the bi-specific antibody or antibody fragment. In a preferred embodiment, the epitope is a hapten. In an alternative

embodiment, the epitope is a part of the carrier. Examples of recognizable haptens include, but are not limited to, chelators, such as DTPA, fluorescein isothiocyanate, vitamin B-12 and other moieties to which specific antibodies can be raised. The carrier portion also may be conjugated to a variety of agents
5 useful for treating or identifying diseased tissue. Examples of conjugated agents include, but are not limited to, metal chelate complexes, drugs, toxins and other effector molecules, such as cytokines, lymphokines, chemokines, immunomodulators, radiosensitizers, asparaginase, carboranes and radioactive halogens. Additionally, enzymes useful for activating a prodrug or increasing
10 the target-specific toxicity of a drug can be conjugated to the carrier. Thus, the use of bsAb which have at least one arm that specifically binds a targetable conjugate allows a variety of therapeutic and diagnostic applications to be performed without raising new bsAb for each application.

The present invention encompasses antibodies and antibody fragments.
15 The antibody fragments are antigen binding portions of an antibody, such as $F(ab')_2$, $F(ab)_2$, Fab' , Fab , and the like. The antibody fragments bind to the same antigen that is recognized by the intact antibody. For example, an anti-CD22 monoclonal antibody fragment binds to an epitope of CD22. The bsAb of the present invention include, but are not limited to, $IgG \times IgG$, $IgG \times F(ab')_2$, IgG
20 $\times Fab'$, $IgG \times scFv$, $F(ab')_2 \times F(ab')_2$, $Fab' \times F(ab')_2$, $Fab' \times Fab'$, $Fab' \times scFv$ and $scFv \times scFv$ bsmabs. Also, species such as $scFv \times IgG \times scFv$ and $Fab' \times IgG \times Fab'$, $scFv \times F(ab')_2 \times scFv$ and $Fab' \times F(ab')_2 \times Fab'$ are included. Most preferably, site-specific attachment sites on the IgG or $F(ab')_2$ of one or both
25 mabs can be utilized, such as an engineered carbohydrate or an engineered or liberated free thiol group. Since these mabs are dimeric they can be coupled with two moles of the second mab. For instance, anti-CEA $F(ab')_2$ mAb having engineered light-chain carbohydrate can be oxidized and converted using a hydrazide-maleimide cross-linker to a derivatized anti-CEA $F(ab')_2$ having at
30 least one pendant maleimide group per each light chain. This species is coupled to an anti-chelate $Fab'-SH$ at a 1:2 molar ratio, at least, such that an anti-chelate- $Fab' \times$ anti-CEA- $F(ab')_2$ -anti-chelate Fab' conjugate is produced. The resultant bsAb is bivalent with respect to the target tissue and the targetable conjugate. It is further understood that the use of the term "bsAb" in the

present disclosure encompasses bi-specific monoclonal antibodies and antibody fragments.

The term "antibody fragment" also includes any synthetic or genetically engineered protein that acts like an antibody by binding to a specific antigen to form a complex. For example, antibody fragments include isolated fragments, "Fv" fragments, consisting of the variable regions of the heavy and light chains, recombinant single chain polypeptide molecules in which light and heavy chain variable regions are connected by a peptide linker ("sFv proteins"), and minimal recognition units consisting of the amino acid residues that mimic the hypervariable region.

The targetable conjugate can be of diverse structure, but is selected not only to elicit sufficient immune responses, but also for rapid *in vivo* clearance when used within the bsAb targeting method. Hydrophobic agents are best at eliciting strong immune responses, whereas hydrophilic agents are preferred for rapid *in vivo* clearance, thus, a balance between hydrophobic and hydrophilic needs to be established. This may be accomplished in a preferred approach, in part, by relying on the use of hydrophilic chelating agents to offset the inherent hydrophobicity of many organic moieties. Also, sub-units of the targetable conjugate may be chosen which have opposite solution properties, for example, peptides, which contain amino acids, some of which are hydrophobic and some of which are hydrophilic. Aside from peptides, carbohydrates may be used. Additionally, the targetable conjugate can comprise PEG derivatives to increase its circulation time in a patient.

Carriers having as few as one amine residue may be used, preferably two to ten amino acid residues, if also coupled to other moieties such as chelating agents. Examples include modified amino acids, such as bis-DTPA-lysine, and bis-DTPA-diamine. These agents can be linked covalently to molecules which are to be targeted. The hapten moiety of the carrier portion should be a low molecular weight conjugate, preferably having a molecular weight of 100,000 daltons or less, and advantageously less than about 20,000 daltons, 10,000 daltons or 5,000 daltons, including the metal ions in the chelates. For instance, the known peptide di-indium-DTPA-Tyr-Lys(DTPA)-OH has been used to generate antibodies against the indium-DTPA portion of the molecule.

However, by use of the non-indium-containing molecule, and appropriate screening steps, new Abs against the tyrosyl-lysine dipeptide can be made. More usually, the antigenic peptide will have four or more residues, such as the peptide Ac-Phe-Lys(DTPA)-Tyr-Lys(DTPA)-NH₂. Again, the non-metal-
5 containing peptide is used as an immunogen, with resultant Abs screened for reactivity against the Phe-Lys-Tyr-Lys backbone.

In one embodiment, unnatural amino acids, e.g., D-amino acids, are incorporated into the backbone structure to ensure that, when used with the final bsAb/targetable conjugate system, the arm of the bsAb which recognizes the
10 targetable conjugate is completely specific.

In a preferred embodiment, the immunogen used has repeating units, resulting in retention of bivalency when used in the final bsAb/targetable conjugate system. Such an immunogen is a peptide of the formula X-Gly-D-Tyr-D-trp-Gly-D-Lys(X)-Gly-D-Tyr-D-Trp-OH, wherein the X-residues are free
15 amino groups which can be substituted later with chelates. X can also represent a moiety selected from the group consisting of a protected amino acid group, a chelating agent and a metal-chelate complex. Thiol groups also may be used for later chelate coupling, in lieu of amino groups, by incorporating cysteinyl-residues into the immunogenic peptide, for example, Ac-Cys(Y)-D-Tyr-D-Trp-Gly-D-Cys(Y)-Gly-D-Tyr-D-Trp-OH, wherein -Y- is a free thiol group. Y can
20 also represent a moiety selected from the group consisting of a protected thiol group, a thiol-linked chelating agent and a metal-chelate complex. The cleaved peptide containing methyl-derivatized thiol groups is for the production of antibodies. Later, the peptide may be prepared with removable protecting
25 groups, such as trityl or acetamidomethyl, for chelate substitution reactions.

The peptides to be used as immunogens are synthesized conveniently on an automated peptide synthesizer using a solid-phase support and standard techniques of repetitive orthogonal deprotection and coupling. Free amino groups in the peptide, that are to be used later for chelate conjugation, are
30 advantageously blocked with small organic moieties, for example by acetylation. For instance, Ac-Gly-D-Tyr-D-Trp-Gly-D-Lys(Ac)-Gly-D-Tyr-D-Trp-OH, cleaved from its assembly resin is then activated through its single carboxyl moiety using active ester/anhydride methodology and coupled in multiple units

to KLH. For immunogenic use, the di-cysteinyl-containing peptide Ac-Cys(Y)-D-Tyr-D-Trp-Gly-D-Cys(Y)-Gly-D-Tyr-D-Trp-OH can be removed from the resin with the thiol groups protected by methylation to generate Ac-Cys(Me)-D-Tyr-D-Trp-Gly-D-Cys(Me)-Gly-D-Tyr-D-Trp-OH. This can then be activated
5 for KLH coupling using the same standard methods. When the peptides are prepared for later use within the bsAb system, they are advantageously cleaved from the resins to generate the corresponding C-terminal amides, in order to inhibit *in vivo* carboxypeptidase activity.

According to one embodiment of the invention, the targetable conjugate
10 can encompass a carbohydrate. Suitable such carbohydrates include carbohydrate chains of two to six sugar units long. The targetable conjugate also can comprise a polymeric carbohydrate, such as dextran.

In another embodiment of the invention, the haptens of the targetable conjugate comprise a known immunogenic recognition moiety, for example, a
15 known hapten. Using a known hapten, for example, fluorescein isothiocyanate (FITC), higher specificity of the targetable conjugate for the antibody is exhibited. This occurs because antibodies raised to the hapten are known and can be incorporated into the inventive antibody. Thus, binding of the targetable conjugate with the attached chelator or metal-chelate complex would be highly
20 specific for the inventive antibody or antibody fragment. Another example of a hapten to be substituted onto the targetable conjugate includes vitamin B12. The use of vitamin B12 is advantageous since anti-B12 Mabs are known and no free serum B12 exists, therefore, great specificity for the antibody may be exhibited. An example of a targetable conjugate containing a hapten includes Ac-Cys-(S-
25 Bz-DTPA)-Gly-Lys-(N-FITC)-Tyr-Cys-(S-Bz-DTPA)NH₂. The chelator or its chelate with a metal cation also can function as the hapten to which an antibody is raised. Another example of a hapten to be conjugated to a targetable conjugate includes biotin.

In a further preferred embodiment, a radionuclide used for imaging
30 and/or therapy may be integrated into the design of the original immunogen. For instance, Ac-Gly-D-iodo-Tyr-D-Trp-Gly-D-Lys(Ac)-Gly-D-iodo-Tyr-D-Trp-OH is used as an immunogen with the express purpose of raising an antibody which is reactive with an iodine-containing peptide, but not with the

non-iodo version of the same peptide, namely Ac-Gly-D-Tyr-D-Trp-Gly-D-Lys(Ac)-Gly-D-Tyr-D-Trp-OH. Specificity of Abs for the former over the latter can be demonstrated using standard screening techniques. Of particular importance within this embodiment is the use of astatine-substituted peptides as immunogens to generate Abs and thus bsAb which recognize peptides substituted with alpha-particle-emitting astatine nuclides for RAIT. In other embodiments, any halogen can be integrated into the design of the original immunogen, including, for example, fluorine-18, bromine, and nuclides of iodine, for example, iodine-124 and iodine-123. Similarly, other non metals can be used, for example ^{32}P , ^{33}P and ^{35}S .

New Abs to peptide backbones are generated by well-known methods for Ab production. For example, injection of an immunogen, such as (peptide)_n-KLH (n=1-30) in complete Freund's adjuvant, followed by two subsequent injections of the same immunogen suspended in incomplete Freund's adjuvant into immunocompetent animals, is followed three days after an i.v. boost of antigen, by spleen cell harvesting. Harvested spleen cells are then fused with Sp2/0-Ag14 myeloma cells and culture supernatants of the resulting clones analyzed for anti-peptide reactivity using a direct-binding ELISA. Fine specificity of generated Abs can be analyzed for by using peptide fragments of the original immunogen. These fragments can be prepared readily using an automated peptide synthesizer. For Ab production, enzyme-deficient hybridomas are isolated to enable selection of fused cell lines. This technique also can be used to raise antibodies to one or more of the chelates comprising the targetable conjugate, e.g., In(III)-DTPA chelates. Monoclonal mouse antibodies to an In(III)-di-DTPA are known (Barbet '395 *supra*).

After the initial raising of antibodies to the immunogen, the antibodies can be sequenced and subsequently prepared by recombinant techniques. Humanization and chimerization of murine antibodies and antibody fragments are well known to those skilled in the art. For example, humanized monoclonal antibodies are produced by transferring mouse complementary determining regions from heavy and light variable chains of the mouse immunoglobulin into a human variable domain, and then, substituting human residues in the framework regions of the murine counterparts. The use of antibody components derived from

humanized monoclonal antibodies obviates potential problems associated with the immunogenicity of murine constant regions. General techniques for cloning murine immunoglobulin variable domains are described, for example, by the publication of Orlandi *et al.*, *Proc. Nat'l Acad. Sci. USA* 86: 3833 (1989), which is incorporated by reference in its entirety. Techniques for producing humanized Mabs are described, for example, by Jones *et al.*, *Nature* 321: 522 (1986), Riechmann *et al.*, *Nature* 332: 323 (1988), Verhoeyen *et al.*, *Science* 239: 1534 (1988), Carter *et al.*, *Proc. Nat'l Acad. Sci. USA* 89: 4285 (1992), Sandhu, *Crit. Rev. Biotech.* 12: 437 (1992), and Singer *et al.*, *J. Immun.* 150: 2844 (1993), each of which is hereby incorporated by reference.

Alternatively, fully human antibodies can be obtained from transgenic non-human animals. See, e.g., Mendez *et al.*, *Nature Genetics*, 15: 146-156 (1997); U.S. Patent No. 5,633,425. For example, human antibodies can be recovered from transgenic mice possessing human immunoglobulin loci. The mouse humoral immune system is humanized by inactivating the endogenous immunoglobulin genes and introducing human immunoglobulin loci. The human immunoglobulin loci are exceedingly complex and comprise a large number of discrete segments which together occupy almost 0.2% of the human genome. To ensure that transgenic mice are capable of producing adequate repertoires of antibodies, large portions of human heavy- and light-chain loci must be introduced into the mouse genome. This is accomplished in a stepwise process beginning with the formation of yeast artificial chromosomes (YACs) containing either human heavy- or light-chain immunoglobulin loci in germline configuration. Since each insert is approximately 1 Mb in size, YAC construction requires homologous recombination of overlapping fragments of the immunoglobulin loci. The two YACs, one containing the heavy-chain loci and one containing the light-chain loci, are introduced separately into mice via fusion of YAC-containing yeast spheroblasts with mouse embryonic stem cells. Embryonic stem cell clones are then microinjected into mouse blastocysts. Resulting chimeric males are screened for their ability to transmit the YAC through their germline and are bred with mice deficient in murine antibody production. Breeding the two transgenic strains, one containing the human heavy-chain loci and the other containing the

human light-chain loci, creates progeny which produce human antibodies in response to immunization.

Unrearranged human immunoglobulin genes also can be introduced into mouse embryonic stem cells via microcell-mediated chromosome transfer (MMCT). See, e.g., Tomizuka *et al.*, *Nature Genetics*, 16: 133 (1997). In this methodology microcells containing human chromosomes are fused with mouse embryonic stem cells. Transferred chromosomes are stably retained, and adult chimeras exhibit proper tissue-specific expression.

As an alternative, an antibody or antibody fragment of the present invention may be derived from human antibody fragments isolated from a combinatorial immunoglobulin library. See, e.g., Barbas *et al.*, *METHODS: A Companion to Methods in Enzymology* 2: 119 (1991), and Winter *et al.*, *Ann. Rev. Immunol.* 12: 433 (1994), which are incorporated by reference. Many of the difficulties associated with generating monoclonal antibodies by B-cell immortalization can be overcome by engineering and expressing antibody fragments in *E. coli*, using phage display. To ensure the recovery of high affinity, monoclonal antibodies a combinatorial immunoglobulin library must contain a large repertoire size. A typical strategy utilizes mRNA obtained from lymphocytes or spleen cells of immunized mice to synthesize cDNA using reverse transcriptase. The heavy- and light-chain genes are amplified separately by PCR and ligated into phage cloning vectors. Two different libraries are produced, one containing the heavy-chain genes and one containing the light-chain genes. Phage DNA is isolated from each library, and the heavy- and light-chain sequences are ligated together and packaged to form a combinatorial library. Each phage contains a random pair of heavy- and light-chain cDNAs and upon infection of *E. coli* directs the expression of the antibody chains in infected cells. To identify an antibody that recognizes the antigen of interest, the phage library is plated, and the antibody molecules present in the plaques are transferred to filters. The filters are incubated with radioactively labeled antigen and then washed to remove excess unbound ligand. A radioactive spot on the autoradiogram identifies a plaque that contains an antibody that binds the antigen. Cloning and expression vectors that are useful for producing a human immunoglobulin phage library can be obtained, for example, from STRATAGENE Cloning Systems (La Jolla, CA).

A similar strategy can be employed to obtain high-affinity scFv. See, e.g., Vaughn *et al.*, *Nat. Biotechnol.*, 14: 309-314 (1996). An scFv library with a large repertoire can be constructed by isolating V-genes from non-immunized human donors using PCR primers corresponding to all known V_H , V_K and V_L gene families. Following amplification, the V_K and V_L pools are combined to form one pool. These fragments are ligated into a phagemid vector. The scFv linker, (Gly₄, Ser)₃, is then ligated into the phagemid upstream of the V_L fragment. The V_H and linker- V_L fragments are amplified and assembled on the J_H region. The resulting V_H -linker- V_L fragments are ligated into a phagemid vector. The phagemid library can be panned using filters, as described above, or using immunotubes (Nunc; Maxisorp). Similar results can be achieved by constructing a combinatorial immunoglobulin library from lymphocytes or spleen cells of immunized rabbits and by expressing the scFv constructs in *P. pastoris*. See, e.g., Ridder *et al.*, *Biotechnology*, 13: 255-260 (1995). Additionally, following isolation of an appropriate scFv, antibody fragments with higher binding affinities and slower dissociation rates can be obtained through affinity maturation processes such as CDR3 mutagenesis and chain shuffling. See, e.g., Jackson *et al.*, *Br. J. Cancer*, 78: 181-188 (1998); Osbourn *et al.*, *Immunotechnology*, 2: 181-196 (1996).

The bsAb can be prepared by techniques known in the art, for example, an anti-CEA tumor Ab and an anti-peptide Ab are both separately digested with pepsin to their respective $F(ab')_2$ s. The anti-CEA-Ab- $F(ab')_2$ is reduced with cysteine to generate Fab' monomeric units which are further reacted with the cross-linker bis(maleimido) hexane to produce Fab'-maleimide moieties. The anti-peptide Ab- $F(ab')_2$ is reduced with cysteine and the purified, recovered anti-peptide Fab'-SH reacted with the anti-CEA-Fab'-maleimide to generate the Fab' x Fab' bi-specific Ab. Alternatively, the anti-peptide Fab'-SH fragment may be coupled with the anti-CEA $F(ab')_2$ to generate a $F(ab')_2$ x Fab' construct, or with anti-CEA IgG to generate an IgG x Fab' bi-specific construct. In one embodiment, the IgG x Fab' construct can be prepared in a site-specific manner by attaching the anti-peptide Fab' thiol group to anti-CEA IgG heavy-chain carbohydrate which has been periodate-oxidized, and subsequently activated by reaction with a commercially available hydrazide-maleimide cross-linker. The

component Abs used can be chimerized or humanized by known techniques. A chimeric antibody is a recombinant protein that contains the variable domains and complementary determining regions derived from a rodent antibody, while the remainder of the antibody molecule is derived from a human antibody.

- 5 Humanized antibodies are recombinant proteins in which murine complementarity determining regions of a monoclonal antibody have been transferred from heavy and light variable chains of the murine immunoglobulin into a human variable domain.

A variety of recombinant methods can be used to produce bi-specific
10 antibodies and antibody fragments. For example, bi-specific antibodies and antibody fragments can be produced in the milk of transgenic livestock. See, e.g., Colman, A., *Biochem. Soc. Symp.*, 63: 141-147, 1998; U.S. Patent #5,827,690. Two DNA constructs are prepared which contain, respectively, DNA segments encoding paired immunoglobulin heavy and light chains. The
15 fragments are cloned into expression vectors which contain a promoter sequence that is preferentially expressed in mammary epithelial cells. Examples include, but are not limited to, promoters from rabbit, cow and sheep casein genes, the cow α -lactoglobulin gene, the sheep β -lactoglobulin gene and the mouse whey acid protein gene. Preferably, the inserted fragment is flanked on its 3' side by
20 cognate genomic sequences from a mammary-specific gene. This provides a polyadenylation site and transcript-stabilizing sequences. The expression cassettes are coinjected into the pronuclei of fertilized, mammalian eggs, which are then implanted into the uterus of a recipient female and allowed to gestate. After birth, the progeny are screened for the presence of both transgenes by
25 Southern analysis. In order for the antibody to be present, both heavy and light chain genes must be expressed concurrently in the same cell. Milk from transgenic females is analyzed for the presence and functionality of the antibody or antibody fragment using standard immunological methods known in the art. The antibody can be purified from the milk using standard methods known in
30 the art.

A chimeric Ab is constructed by ligating the cDNA fragment encoding the mouse light variable and heavy variable domains to fragment encoding the C domains from a human antibody. Because the C domains do not contribute to

antigen binding, the chimeric antibody will retain the same antigen specificity as the original mouse Ab but will be closer to human antibodies in sequence.

Chimeric Abs still contain some mouse sequences, however, and may still be immunogenic. A humanized Ab contains only those mouse amino acids necessary to recognize the antigen. This product is constructed by building into a human antibody framework the amino acids from mouse complementarity determining regions.

Other recent methods for producing bsAbs include engineered recombinant Abs which have additional cysteine residues so that they crosslink more strongly than the more common immunoglobulin isotypes. See, e.g., FitzGerald *et al.*, Protein Eng. 10(10):1221-1225, 1997. Another approach is to engineer recombinant fusion proteins linking two or more different single-chain antibody or antibody fragment segments with the needed dual specificities. See, e.g., Coloma *et al.*, *Nature Biotech.* 15:159-163, 1997. A variety of bi-specific fusion proteins can be produced using molecular engineering. In one form, the bi-specific fusion protein is monovalent, consisting of, for example, a scFv with a single binding site for one antigen and a Fab fragment with a single binding site for a second antigen. In another form, the bi-specific fusion protein is divalent, consisting of, for example, an IgG with two binding sites for one antigen and two scFv with two binding sites for a second antigen.

Functional bi-specific single-chain antibodies (bscAb), also called diabodies, can be produced in mammalian cells using recombinant methods. See, e.g., Mack *et al.*, *Proc. Natl. Acad. Sci.*, 92: 7021-7025, 1995. For example, bscAb are produced by joining two single-chain Fv fragments via a glycine-serine linker using recombinant methods. The V light-chain (V_L) and V heavy-chain (V_H) domains of two antibodies of interest are isolated using standard PCR methods. The V_L and V_H cDNA's obtained from each hybridoma are then joined to form a single-chain fragment in a two-step fusion PCR. The first PCR step introduces the $(Gly_4-Ser)_3$ linker, and the second step joins the V_L and V_H amplicons. Each single chain molecule is then cloned into a bacterial expression vector. Following amplification, one of the single-chain molecules is excised and sub-cloned into the other vector, containing the second single-chain molecule of interest. The resulting bscAb fragment is subcloned into an

eukaryotic expression vector. Functional protein expression can be obtained by transfecting the vector into chinese hamster ovary cells. Bi-specific fusion proteins are prepared in a similar manner. Bi-specific single-chain antibodies and bi-specific fusion proteins are included within the scope of the present invention.

Bi-specific fusion proteins linking two or more different single-chain antibodies or antibody fragments are produced in similar manner. Recombinant methods can be used to produce a variety of fusion proteins. For example a fusion protein comprising a Fab fragment derived from a humanized monoclonal anti-CEA antibody and a scFv derived from a murine anti-diDTPA can be produced. A flexible linker, such as GGGS connects the scFv to the constant region of the heavy chain of the anti-CEA antibody. Alternatively, the scFv can be connected to the constant region of the light chain of hMN-14. Appropriate linker sequences necessary for the in-frame connection of the heavy chain Fd to the scFv are introduced into the VL and VK domains through PCR reactions. The DNA fragment encoding the scFv is then ligated into a staging vector containing a DNA sequence encoding the CH1 domain. The resulting scFv-CH1 construct is excised and ligated into a vector containing a DNA sequence encoding the VH region of an anti-CEA antibody. The resulting vector can be used to transfect mammalian cells for the expression of the bi-specific fusion protein.

Large quantities of bscAb and fusion proteins can be produced using *Escherichia coli* expression systems. See, e.g., Zhenping *et al.*, *Biotechnology*, 14: 192-196, 1996. A functional bscAb can be produced by the coexpression in *E. coli* of two "cross-over" scFv fragments in which the V_L and V_H domains for the two fragments are present on different polypeptide chains. The V light-chain (V_L) and V heavy-chain (V_H) domains of two antibodies of interest are isolated using standard PCR methods. The cDNA's are then ligated into a bacterial expression vector such that C-terminus of the V_L domain of the first antibody of interest is ligated via a linker to the N-terminus of the V_H domain of the second antibody. Similarly, the C-terminus of the V_L domain of the second antibody of interest is ligated via a linker to the N-terminus of the V_H domain of the first antibody. The resulting dicistronic operon is placed under transcriptional

control of a strong promoter, e.g., the *E. coli* alkaline phosphatase promoter which is inducible by phosphate starvation. Alternatively, single-chain fusion constructs have successfully been expressed in *E. coli* using the *lac* promoter and a medium consisting of 2% glycine and 1% Triton X-100. See, e.g., Yang
5 *et al.*, *Appl. Environ. Microbiol.*, 64: 2869-2874, 1998. An *E. coli*, heat-stable, enterotoxin II signal sequence is used to direct the peptides to the periplasmic space. After secretion, the two peptide chains associate to form a non-covalent heterodimer which possesses both antigen binding specificities. The bscAb is purified using standard procedures known in the art, e.g., Staphylococcal
10 protein A chromatography.

Functional bscAb and fusion proteins also can be produced in the milk of transgenic livestock. See, e.g., Colman, A., *Biochem. Soc. Symp.*, 63: 141-147, 1998; U.S. Patent #5,827,690. The bscAb fragment, obtained as described above, is cloned into an expression vector containing a promoter sequence that
15 is preferentially expressed in mammary epithelial cells. Examples include, but are not limited to, promoters from rabbit, cow and sheep casein genes, the cow α -lactoglobulin gene, the sheep β -lactoglobulin gene and the mouse whey acid protein gene. Preferably, the inserted bscAb is flanked on its 3' side by cognate genomic sequences from a mammary-specific gene. This provides a
20 polyadenylation site and transcript-stabilizing sequences. The expression cassette is then injected into the pronuclei of fertilized, mammalian eggs, which are then implanted into the uterus of a recipient female and allowed to gestate. After birth, the progeny are screened for the presence of the introduced DNA by Southern analysis. Milk from transgenic females is analyzed for the presence
25 and functionality of the bscAb using standard immunological methods known in the art. The bscAb can be purified from the milk using standard methods known in the art. Transgenic production of bscAb in milk provides an efficient method for obtaining large quantities of bscAb.

Functional bscAb and fusion proteins also can be produced in transgenic
30 plants. See, e.g., Fiedler *et al.*, *Biotech.*, 13: 1090-1093, 1995; Fiedler *et al.*, *Immunotechnology*, 3: 205-216, 1997. Such production offers several advantages including low cost, large scale output and stable, long term storage. The bscAb fragment, obtained as described above, is cloned into an expression

vector containing a promoter sequence and encoding a signal peptide sequence, to direct the protein to the endoplasmic recticulum. A variety of promoters can be utilized, allowing the practitioner to direct the expression product to particular locations within the plant. For example, ubiquitous expression in tobacco plants can be achieved by using the strong cauliflower mosaic virus 35S promoter, while organ specific expression is achieved via the seed specific legumin B4 promoter. The expression cassette is transformed according to standard methods known in the art. Transformation is verified by Southern analysis. Transgenic plants are analyzed for the presence and functionality of the bscAb using standard immunological methods known in the art. The bscAb can be purified from the plant tissues using standard methods known in the art.

Additionally, transgenic plants facilitate long term storage of bscAb and fusion proteins. Functionally active scFv proteins have been extracted from tobacco leaves after a week of storage at room temperature. Similarly, transgenic tobacco seeds stored for 1 year at room temperature show no loss of scFv protein or its antigen binding activity.

Functional bscAb and fusion proteins also can be produced in insect cells. See, e.g., Mahiouz *et al.*, *J. Immunol. Methods*, 212: 149-160 (1998). Insect-based expression systems provide a means of producing large quantities of homogenous and properly folded bscAb. The baculovirus is a widely used expression vector for insect cells and has been successfully applied to recombinant antibody molecules. See, e.g., Miller, L.K., *Ann. Rev. Microbiol.*, 42: 177 (1988); Bei *et al.*, *J. Immunol. Methods*, 186: 245 (1995). Alternatively, an inducible expression system can be utilized by generating a stable insect cell line containing the bscAb construct under the transcriptional control of an inducible promoter. See, e.g., Mahiouz *et al.*, *J. Immunol. Methods*, 212: 149-160 (1998). The bscAb fragment, obtained as described above, is cloned into an expression vector containing the *Drosophila* metallothionein promoter and the human HLA-A2 leader sequence. The construct is then transfected into *D. melanogaster* SC-2 cells. Expression is induced by exposing the cells to elevated amounts of copper, zinc or cadmium. The presence and functionality of the bscAb is determined using standard

immunological methods known in the art. Purified bscAb is obtained using standard methods known in the art.

The presence of hydrophilic chelate moieties on the targetable conjugate helps to ensure rapid *in vivo* clearance. In addition to hydrophilicity, chelates are chosen for their metal-binding properties, and are changed at will since, at least for those targetable conjugates whose bsAb epitope is part of the peptide or is a non-chelated hapten, recognition of the metal-chelate complex is no longer an issue. Particularly useful metal-chelate combinations include 2-benzyl-DTPA and its monomethyl and cyclohexyl analogs, used with scandium-47, iron-52, cobalt-55, gallium-67, gallium-68, indium-111, zirconium-89, yttrium-90, terbium-161, lutetium-177, bismuth-212, bismuth-213, and actinium-225 for radio-imaging and RAIT. The same chelators, when complexed with non-radioactive metals, such as manganese, iron and gadolinium can be used for MRI, when used along with the bsAbs of the invention. Macrocyclic chelators such as NOTA, DOTA, and TETA are of use with a variety of metals and radiometals, most particularly with radionuclides of gallium, yttrium and copper, respectively. DTPA and DOTA-type chelators, where the ligand includes hard base chelating functions such as carboxylate or amine groups, are most effective for chelating hard acid cations, especially Group IIa and Group IIIa metal cations. Such metal-chelate complexes can be made very stable by tailoring the ring size to the metal of interest. Other ring-type chelators such as macrocyclic polyethers, which are of interest for stably binding nuclides such as radium-223 for RAIT are encompassed by the invention. Porphyrin chelators may be used with numerous radiometals, and are also useful as certain non-radioactive metal complexes for bsAb-directed immuno-phototherapy. More than one type of chelator may be conjugated to a carrier to bind multiple metal ions, *e.g.*, non-radioactive ions, diagnostic radionuclides and/or therapeutic radionuclides. One example is a bis-¹¹¹In-DTPA conjugate that also bears a DOTA-⁹⁰Y chelate.

Chelators such as those disclosed in U.S. Patent 5,753,206, especially thiosemicarbazonylglyoxylcysteine(TscG-Cys) and thiosemicarbazinyl-acetylcysteine (TscA-Cys) chelators are advantageously used to bind soft acid cations of Tc, Re, Bi and other transition metals, lanthanides and actinides that

are tightly bound to soft base ligands, especially sulfur- or phosphorus-containing ligands. It can be useful to link more than one type of chelator to a peptide, e.g., a DTPA or similar chelator for, say In(III) cations, and a thiol-containing chelator, e.g., TscG-Cys, for Tc cations. Because antibodies to a di-DTPA hapten are known (Barbet '395, *supra*) and are readily coupled to a targeting antibody to form a bsAb, it is possible to use a peptide hapten with non-radioactive diDTPA chelates and another chelate for binding a radioisotope, in a pretargeting protocol, for targeting the radioisotope. One example of such a peptide is Ac-Lys(DTPA)-TyrLys(DTPA)-Lys(TscG-Cys-)-NH₂. This peptide can be preloaded with In(III) and then labeled with 99-m-Tc cations, the In(III) ions being preferentially chelated by the DTPA and the Tc cations binding preferentially to the thiol-containing TscG-CysC. Other hard acid chelators such as NOTA, DOTA, TETA and the like can be substituted for the DTPA groups, and Mabs specific to them can be produced using analogous techniques to those used to generate the anti-di-DTPA Mab.

It will be appreciated that two different hard acid or soft acid chelators can be incorporated into the targetable conjugate, e.g., with different chelate ring sizes, to bind preferentially to two different hard acid or soft acid cations, due to the differing sizes of the cations, the geometries of the chelate rings and the preferred complex ion structures of the cations. This will permit two different metals, one or both of which may be radioactive or useful for MRI enhancement, to be incorporated into a targetable conjugate for eventual capture by a pre-targeted bsAb.

Chelators are coupled to the carrier portion of a targetable conjugate using standard chemistries. For instance, excess 2-(*p*-isothiocyanato)benzyl-DTPA is reacted with peptide NH₂ groups to form thiourea bonds between the *p*-isothiocyanate of the chelator and the free 1- α and 6- ϵ -amino groups of the peptide, when a peptide is the targetable conjugate. Alternatively, the bis-anhydride of DTPA can be coupled directly to a free amine group on the peptide. The desired chelator-peptide is purified chromatographically and is ready for use as a metal binding agent. Similarly, DOTA is mono-activated at one carboxyl group using a carbodiimide, and two DOTA units are coupled to the peptide's free amino-groups. Chelators bearing groups specifically reactive

with thiols are used for reaction with peptides such as Ac-Cys-D-Tyr-D-Trp-Gly-D-Cys-Gly-D-Tyr-D-Trp-NH₂. Such a chelator is exemplified by 2-(p-bromoacetamido)benzyl-DTPA, which may be used to alkylate the peptide's free thiol groups under mild, neutral conditions.

5 Chelator-peptide conjugates may be stored for long periods as solids. They may be metered into unit doses for metal-binding reactions, and stored as unit doses either as solids, aqueous or semi-aqueous solutions, frozen solutions or lyophilized preparations. They may be labeled by well-known procedures. Typically, a hard acid cation is introduced as a solution of a convenient salt, and
10 is taken up by the hard acid chelator and possibly by the soft acid chelator. However, later addition of soft acid cations leads to binding thereof by the soft acid chelator, displacing any hard acid cations which may be chelated therein. For example, even in the presence of an excess of non-radioactive ¹¹¹InCl₃, labeling with ^{99m}Tc(V) glucoheptonate or with Tc cations generated *in situ*
15 with stannous chloride and Na^{99m}TcO₄ proceeds quantitatively on the soft acid chelator. Other soft acid cations such as Re-186, Re-188, Bi-213 and divalent or trivalent cations of Mn, Co, Ni, Pb, Cu, Cd, Au, Fe, Ag (monovalent), Zn and Hg, especially Cu-64 and Cu-67, and the like, some of which are useful for radioimmunodiagnosis or radioimmunotherapy, can be loaded onto the carrier
20 peptide by analogous methods. Re cations also can be generated *in situ* from perrhenate and stannous ions or a prerduced rhenium glucoheptonate or other transchelator can be used. Because reduction of perrhenate requires more stannous ion (typically above 200 ug/mL final concentration) than is needed for the reduction of technetium, extra care needs to be taken to ensure that the
25 higher levels of stannous ion do not reduce sensitive disulfide bonds such as those present in disulfide-cyclized peptides. A convenient way to prepare ReO metal complexes of the TscG-Cys- ligands is by reacting the peptide with ReOCl₃(P(Ph₃)₂) but it is also possible to use other reduced species such as ReO(ethylenediamine)₂.

30 In one embodiment of the present invention, the bsAb is given at some time prior to administration of the therapeutic agent which is associated with the targetable conjugate. The doses and timing of the reagents can be readily worked out by a skilled artisan, and are dependent on the specific nature of the

reagents employed. If a bsAb-F(ab')₂ derivative is given first, then a waiting time of 1-6 days before administration of the targetable conjugate would be appropriate. If an IgG-Fab' bsAb conjugate is the primary targeting vector, then a longer waiting period before administration of the targetable conjugate would be indicated, probably in the range of 3-15 days. If a bi-specific fusion protein, for example an anti-CEA Fab x anti-peptide scFv, is the primary targeting vector, a shorter waiting period before administration of the targetable conjugate would be indicated, probably in the range of 1-5 days.

In another embodiment, the present invention can be used in Boron Neutron Capture Therapy (BNCT) protocols. BNCT is a binary system designed to deliver ionizing radiation to tumor cells by neutron irradiation of tumor-localized boron-10 atoms. BNCT is based on the nuclear reaction which occurs when a stable isotope, isotopically enriched B-10 (present in 19.8% natural abundance), is irradiated with thermal neutrons to produce an alpha particle and a Li-7 nucleus. These particles have a path length of about one cell diameter, resulting in high linear energy transfer. Just a few of the short-range 1.7 MeV alpha particles produced in this nuclear reaction are sufficient to target the cell nucleus and destroy it. Success with BNCT of cancer requires methods for localizing a high concentration of boron-10 at tumor sites, while leaving non-target organs essentially boron-free. Compositions and methods for treating tumors in patients using pre-targeting bsAb for BNCT are described in U.S.S.N. 09/205,243 and can easily be modified in accordance with the present invention. Additionally, other elements are suitable for neutron capture reactions. One example is uranium. Uranium, in large amounts, can be bound by naturally occurring chelating agents such as ferritin. Such strategies have been described in U.S.S.N. **, are easily adaptable to the present invention and are hereby incorporated in their entirety by reference.

In another embodiment of the practice of the invention, the bsAb is administered prior to administration of a diagnostic agent which is associated with the targetable conjugate. After sufficient time has passed for the bsAb to target to the diseased tissue, the diagnostic agent is administered. Subsequent to administration of the diagnostic agent, imaging can be performed. Tumors can be detected in body cavities by means of directly or indirectly viewing various

structures to which light is delivered and then collected. Lesions at any body site can be viewed so long as nonionizing radiation can be delivered and recaptured from these structures. For example, positron emission tomography (PET) which is a high resolution, non-invasive, imaging technique can be used with the
5 inventive antibodies for the visualization of human disease. In PET, 511 keV gamma photons produced during positron annihilation decay are detected. Similar pre-targeting strategies for PET using Fluorine-18 and Gallium-68 have been described, respectively in U.S.S.N. 09/146,318 and (second serial number is pending). The methodologies described in these applications are easily
10 adaptable to the present invention and are hereby incorporated in their entirety by reference.

As another example, the present inventive antibodies or antibody fragments can be used in a method of photodynamic diagnosis or therapy. In a diagnostic method, a diagnostic agent is injected, for example, systemically, and laser-
15 induced fluorescence can be used by endoscopes to detect sites of cancer which have accreted the light-activated agent. For example, this has been applied to fluorescence bronchoscopic disclosure of early lung tumors (Doiron *et al.*, *Chest* 76:32, 1979, incorporated herein by reference; and references cited above). In another example, the inventive antibodies and antibody fragments can be used in
20 single photon emission. For example, a Tc-99m-labeled diagnostic agent can be administered to a patient following administration of the inventive antibodies or antibody fragments. The patient is then scanned with a gamma camera which produces single-photon emission computed tomographic images and defines the lesion or tumor site.

25 The present invention also can be used in a method for photodynamic therapy. In this methodology, a photosensitizer, for example a hematoporphyrin derivative such as dihematoporphyrin ether, is administered to a patient. Anti-tumor activity is initiated by the use of strong red light, for example, at 630 nanometers wavelength. Alternate photosensitizers can be utilized, including
30 those useful at longer wavelengths, where skin is less photosensitized by the sun. Examples of such photosensitizers include, but are not limited to, benzoporphyrin monoacid ring A (BPD-MA), tin etiopurpurin (SnET2), sulfonated aluminum phthalocyanine (AlSPc) and lutetium texaphyrin (Lutex).

In other embodiments of the present invention, the carrier portion of the targetable conjugate can be conjugated to an enzyme capable of activating a prodrug at the target site or improving the efficacy of a normal therapeutic by controlling the body's detoxification pathways. Following administration of the bsAb, an enzyme conjugated to the carrier is administered. After the enzyme is pre-targeted to the target site, a cytotoxic drug is injected, which is known to act at the target site, or a prodrug form thereof which is converted to the drug *in situ* by the pre-targeted enzyme. The drug is one which is detoxified to form an intermediate of lower toxicity, most commonly a glucuronide, using the mammal's ordinary detoxification processes. The detoxified intermediate, *e.g.*, the glucuronide, is reconverted to its more toxic form by the pre-targeted enzyme and thus has enhanced cytotoxicity at the target site. This results in a recycling of the drug. Similarly, an administered prodrug can be converted to an active drug through normal biological processes. The pre-targeted enzyme improves the efficacy of the treatment by recycling the detoxified drug. This approach can be adopted for use with any enzyme-drug pair. Similar pre-targeting strategies have been described in U.S.S.N. 60/101,039. Those methodologies are easily adaptable to the present invention and are hereby incorporated in their entirety by reference.

In an alternative embodiment, the enzyme-carrier conjugate can be mixed with the targeting bsAb prior to administration to the patient. After a sufficient time has passed for the enzyme-carrier-bsAb conjugate to localize to the target site and for unbound conjugate to clear from circulation, a prodrug is administered. As discussed above, the prodrug is then converted to the drug *in situ* by the pre-targeted enzyme.

Certain cytotoxic drugs that are useful for anticancer therapy are relatively insoluble in serum. Some are also quite toxic in an unconjugated form, and their toxicity is considerably reduced by conversion to prodrugs. Conversion of a poorly soluble drug to a more soluble conjugate, *e.g.*, a glucuronide, an ester of a hydrophilic acid or an amide of a hydrophilic amine, will improve its solubility in the aqueous phase of serum and its ability to pass through venous, arterial or capillary cell walls and to reach the interstitial fluid bathing the tumor. Cleavage of the prodrug deposits the less soluble drug at the

target site. Many examples of such prodrug-to-drug conversions are disclosed in Hansen U.S.S.N. 08/445,110.

Conversion of certain toxic substances such as aromatic or alicyclic alcohols, thiols, phenols and amines to glucuronides in the liver is the body's method of detoxifying them and making them more easily excreted in the urine. One type of anti-tumor drug that can be converted to such a substrate is epirubicin, a 4-epimer of doxorubicin (Adriamycin), which is an anthracycline glycoside and has been shown to be a substrate for human beta-D-glucuronidase See, e.g., Arcamone, *Cancer Res.*, 45:5995, 1985. Other analogues with fewer polar groups are expected to be more lipophilic and show greater promise for such an approach. Other drugs or toxins with aromatic or alicyclic alcohol, thiol or amine groups are candidates for such conjugate formation. These drugs, or other prodrug forms thereof, are suitable candidates for the site-specific enhancement methods of the present invention.

The prodrug CPT-11 (irinotecan) is converted *in vivo* by carboxylesterase to the active metabolite SN-38. SN-38 is a highly effective anti-tumor agent; however, therapeutic doses can not be administered to patients due to its toxicity. One application of the invention, therefore, is to target such therapies to the tumor site using a bsAb specific for a tumor-associated antigen and a hapten (e.g. di-DTPA) followed by injection of a di-DTPA-carboxylesterase conjugate. Once a suitable tumor-to-background localization ratio has been achieved, the CPT-11 is given and the tumor-localized carboxylesterase serves to convert CPT-11 to SN-38 at the tumor. Due to its poor solubility, the active SN-38 will remain in the vicinity of the tumor and, consequently, will exert an effect on adjacent tumor cells that are negative for the antigen being targeted. This is a further advantage of the method. Modified forms of carboxylesterases have been described and are within the scope of the invention. See, e.g., Potter *et al.*, *Cancer Res.*, 58:2646-2651 and 3627-3632, 1998.

Etoposide is a widely used cancer drug that is detoxified to a major extent by formation of its glucuronide and is within the scope of the invention. See, e.g., Hande *et al.*, *Cancer Res.*, 48: 1829-1834, 1988. Glucuronide conjugates can be prepared from cytotoxic drugs and can be injected as

therapeutics for tumors pre-targeted with mAb-glucuronidase conjugates. See, e.g., Wang *et al.*, *Cancer Res.*, 52:4484-4491, 1992. Accordingly, such conjugates also can be used with the pre-targeting approach described here. Similarly, designed prodrugs based on derivatives of daunomycin and doxorubicin have been described for use with carboxylesterases and glucuronidases. See, e.g., Bakina *et al.*, *J. Med Chem.*, 40:4013-4018, 1997. Other examples of prodrug/enzyme pairs that can be used within the present invention include, but are not limited to, glucuronide prodrugs of hydroxy derivatives of phenol mustards and beta-glucuronidase; phenol mustards or CPT-11 and carboxypeptidase; methotrexate-substituted alpha-amino acids and carboxypeptidase A; penicillin or cephalosporin conjugates of drugs such as 6-mercaptopurine and doxorubicin and beta-lactamase; etoposide phosphate and alkaline phosphatase.

In other embodiments of the present invention, the enzyme capable of activating a prodrug at the target site or improving the efficacy of a normal therapeutic by controlling the body's detoxification pathways is conjugated to the hapten. The enzyme-hapten conjugate is administered to the patient following administration of the pre-targeting bsAb and is directed to the target site. After the enzyme is localized at the target site, a cytotoxic drug is injected, which is known to act at the target site, or a prodrug form thereof which is converted to the drug *in situ* by the pre-targeted enzyme. As discussed above, the drug is one which is detoxified to form an intermediate of lower toxicity, most commonly a glucuronide, using the mammal's ordinary detoxification processes. The detoxified intermediate, e.g., the glucuronide, is reconverted to its more toxic form by the pre-targeted enzyme and thus has enhanced cytotoxicity at the target site. This results in a recycling of the drug. Similarly, an administered prodrug can be converted to an active drug through normal biological processes. The pre-targeted enzyme improves the efficacy of the treatment by recycling the detoxified drug. This approach can be adopted for use with any enzyme-drug pair. In an alternative embodiment, the enzyme-hapten conjugate can be mixed with the targeting bsAb prior to administration to the patient. After a sufficient time has passed for the enzyme-hapten-bsAb conjugate to localize to the target site and for unbound conjugate to clear from

circulation, a prodrug is administered. As discussed above, the prodrug is then converted to the drug *in situ* by the pre-targeted enzyme.

In another embodiment of the present invention, the carrier portion of the targetable conjugate is conjugated to a prodrug. The pre-targeting bsAb is administered to the patient and allowed to localize to the target and substantially clear circulation. At an appropriate later time, a targetable conjugate comprising a prodrug, for example poly-glutamic acid (SN-38-ester)₁₀, is given, thereby localizing the prodrug specifically at the tumor target. It is known that tumors have increased amounts of enzymes released from intracellular sources due to the high rate of lysis of cells within and around tumors. A practitioner can capitalize on this fact by appropriately selecting prodrugs capable of being activated by these enzymes. For example, carboxylesterase activates the prodrug poly-glutamic acid (SN-38-ester)₁₀ by cleaving the ester bond of the poly-glutamic acid (SN-38-ester)₁₀ releasing large concentrations of free SN-38 at the tumor. Alternatively, the appropriate enzyme also can be targeted to the tumor site.

After cleavage from the targetable conjugate, the drug is internalized by the tumor cells. Alternatively, the drug can be internalized as part of an intact complex by virtue of cross-linking at the target. The targetable conjugate can induce internalization of tumor-bound bsAb and thereby improve the efficacy of the treatment by causing higher levels of the drug to be internalized.

A variety of carriers are well-suited for conjugation to prodrugs, including polyamino acids, such as polylysine, polyglutamic (E) and aspartic acids (D), including D-amino acid analogs of the same, co-polymers, such as poly(Lys-Glu) {poly[KE]}, advantageously from 1:10 to 10:1. Copolymers based on amino acid mixtures such as poly(Lys-Ala-Glu-Tyr) (KAey; 5:6:2:1) can also be employed. Smaller polymeric carriers of defined molecular weight can be produced by solid-phase peptide synthesis techniques, readily producing polypeptides of from 2-50 residues in chain length. A second advantage of this type of reagent, other than precise structural definition, is the ability to place single or any desired number of chemical handles at certain points in the chain. These can be used later for attachment of recognition and therapeutic haptens at chosen levels of each moiety.

Poly(ethylene) glycol [PEG] has desirable *in vivo* properties for a bi-specific antibody prodrug approach. Ester linkages between the hydroxyl group of SN-38 and both ends of a standard di-hydroxyl PEG can be introduced by insertion of diacids such as succinic acid between the SN-38 and PEG hydroxyl groups, to generate species such as SN-38-O-CO(CH₂)₂CO-O-PEG-0-CO(CH₂)₂CO-OSN-38. The di-SN-38-PEG produced can be considered as the shortest member of the class of SN-38-polymer prodrugs. The desirable *in vivo* properties of PEG derivatives and the limited loading capacity due to their dimeric functionality led to the preparation of PEG co-polymers having greater hapten-bearing capacity such as those described by Poiani *et al.* See, e.g., Poiani *et al. Bioconjugate Chem.*, 5:621-630, 1994. PEG derivatives are activated at both ends as their bis(succinimidyl)carbonate derivatives and co-polymerized with multi-functional diamines such as lysine. The product of such co-polymerization, containing (-Lys(COOH)-PEG-Lys(COOH)-PEG-) _n repeat units wherein the lysyl carboxyl group is not involved in the polymerization process, can be used for attachment of SN-38 residues. The SN-38 residues are reacted with the free carboxyl groups to produce SN-38 esters of the (-Lys(COOH)-PEG-Lys(COOH)-PEG-) _n chain.

Other synthetic polymers that can be used to carry recognition haptens and prodrugs include N-(2-hydroxypropyl)methacrylamide (HMPA) copolymers, poly(styrene-co-maleic acid/anhydride (SMA), poly(divinylether maleic anhydride) (DIVEMA), polyethyleneimine, ethoxylated polyethyleneimine, starburst dendrimers and poly(N-vinylpyrrolidone) (PVP). As an example, DIVEMA polymer comprised of multiple anhydride units is reacted with a limited amount of SN-38 to produce a desired substitution ratio of drug on the polymer backbone. Remaining anhydride groups are opened under aqueous conditions to produce free carboxylate groups. A limited number of the free carboxylate groups are activated using standard water-soluble peptide coupling agents, e.g. 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), and coupled to a recognition moiety bearing a free amino group. An example of the latter is histamine, to which antibodies have been raised in the past.

A variety of prodrugs can be conjugated to the carrier portion of the targetable conjugate. The above exemplifications of polymer use are concerned with SN-38, the active metabolite of the prodrug CPT-11 (irinotecan). SN-38 has an aromatic hydroxyl group that was used in the above descriptions to produce aryl esters susceptible to esterase-type enzymes. Similarly the camptothecin analog topotecan, widely used in chemotherapy, has an available aromatic hydroxyl residue that can be used in a similar manner as described for SN-38, producing esterase-susceptible polymer-prodrugs.

Doxorubicin also contains aromatic hydroxyl groups that can be coupled to carboxylate-containing polymeric carriers using acid-catalyzed reactions similar to those described for the camptothecin family. Similarly, doxorubicin analogs like daunomycin, epirubicin and idarubicin can be coupled in the same manner. Doxorubicin and other drugs with amino 'chemical handles' active enough for chemical coupling to polymeric carriers can be effectively coupled to carrier molecules via these free amino groups in a number of ways. Polymers bearing free carboxylate groups can be activated *in situ* (EDC) and the activated polymers mixed with doxorubicin to directly attach the drug to the side-chains of the polymer via amide bonds. Amino-containing drugs can also be coupled to amino-pendant polymers by mixing commercially available and cleavable cross-linking agents, such as ethylene glycobis(succinimidylsuccinate) (EGS, Pierce Chemical Co., Rockford, IL) or bis-[2-(succinimido-oxycarbonyloxy)ethyl]sulfone (BSOCOES, Molecular Biosciences, Huntsville, AL), to cross-link the two amines as two amides after reaction with the bis(succinimidyl) ester groups. This is advantageous as these groups remain susceptible to enzymatic cleavage. For example, (doxorubicin-EGS)_n-poly-lysine remains susceptible to enzymatic cleavage of the diester groups in the EGS linking chain by enzymes such as esterases. Doxorubicin also can be conjugated to a variety of peptides, for example, HyBnK(DTPA)YK(DTPA)-NH₂, using established procedures (HyBn= p-H₂NNHC₆H₄CO₂H). See Kaneko *et al.*, *J. Bioconjugate Chem.*, 2: 133-141, 1991.

Methotrexate also has an available amino group for coupling to activated carboxylate-containing polymers, in a similar manner to that described for doxorubicin. It also has two glutamyl carboxyl groups (alpha and gamma) that

can be activated for coupling to amino-group containing polymers. The free carboxylate groups of methotrexate can be activated *in situ* (EDC) and the activated drug mixed with an amino-containing polymer to directly attach the drug to the side-chains of the polymer via amide bonds. Excess unreacted or
5 cross-reacted drug is separated readily from the polymer-drug conjugate using size-exclusion or ion-exchange chromatography.

Maytansinoids and calicheamicins (such as esperamycin) contain mixed di- and tri-sulfide bonds that can be cleaved to generate species with a single thiol useful for chemical manipulation. The thiomaytensinoid or thioespera-
10 mycin is first reacted with a cross-linking agent such as a maleimido-peptide that is susceptible to cleavage by peptidases. The C-terminus of the peptide is then activated and coupled to an amino-containing polymer such as polylysine.

In still other embodiments, the bi-specific antibody-directed delivery of therapeutics or prodrug polymers to *in vivo* targets can be combined with bi-
15 specific antibody delivery of radionuclides, such that combination chemotherapy and radioimmunotherapy is achieved. Each therapy can be conjugated to the targetable conjugate and administered simultaneously, or the nuclide can be given as part of a first targetable conjugate and the drug given in a later step as part of a second targetable conjugate. In one simple embodiment, a peptide
20 containing a single prodrug and a single nuclide is constructed. For example, the tripeptide Ac-Glu-Gly-Lys-NH₂ can be used as a carrier portion of a targetable conjugate, whereby SN-38 is attached to the gamma glutamyl carboxyl group as an aryl ester, while the chelate DOTA is attached to the epsilon amino group as an amide, to produce the complex Ac-Glu(SN-38)-Gly-
25 Lys(DOTA)-NH₂. The DOTA chelate can then be radiolabeled with various metals for imaging and therapy purposes including In-111, Y-90, Sm-153, Lu-177 and Zr-89. As the metal-DOTA complex may represent the recognizable hapten on the targetable conjugate, the only requirement for the metal used as part of the DOTA complex is that the secondary recognition antibody also used
30 recognizes that particular metal-DOTA complex at a sufficiently high affinity. Generally, this affinity (log K_a) is between 6-11. Polymeric peptides such as poly[Glu(SN-38)₁₀-Lys(Y-90-DOTA)₂] can be given as readily as the more chemically defined lower MW reagent above, and are indeed preferred. Also,

triply substituted polymers can be used, such as poly[Glu(Sn-38)₁₀-Lys(Y-90-DOTA)_n(histamine-succinate)_m], where n and m are integers, such that the recognition agent is independent of the radioimmunotherapy agent. The prodrug is activated by carboxylesterases present at the tumor site or by

- 5 carboxylesterases targeted to the site using a second targetable conjugate.

Alternatively, a combination therapy can be achieved by administering the chemotherapy and radioimmunotherapy agents in separate steps. For example, a patient expressing CEA-tumors is first administered bsAb with at least one arm which specifically binds CEA and at least one other arm which

10 specifically binds the targetable conjugate whose hapten is a conjugate of yttrium-DOTA. Later the patient is treated with a targetable conjugate comprising a conjugate of yttrium-DOTA-beta-glucuronidase. After sufficient time for bsAb and enzyme localization and clearance, a second targetable conjugate, comprising Ac-Glu(SN-38)-Gly-Lys(Y-90-DOTA)-NH₂, is given.

15 The second targetable conjugate localizes to the tumor by virtue of bsAb at the tumor that are not already bound to a first targetable conjugate. First targetable conjugates which are localized to the target site act on the Ac-Glu(SN-38)-Gly-Lys(Y-90-DOTA)-NH₂ to liberate the free SN-38 drug. Localization of both the prodrug and its respective enzyme to the target site enhances the production of

20 active drug by ensuring that the enzyme is not substrate limited. This embodiment constitutes a marked improvement of current prodrug methodologies currently practiced in the art.

Another advantage of administering the prodrug-polymer in a later step, after the nuclide has been delivered as part of a previously given targetable

25 conjugate, is that the synergistic effects of radiation and drug therapy can be manipulated and, therefore, maximized. It is hypothesized that tumors become more 'leaky' after RAIT due to radiation damage. This can allow a polymer-prodrug to enter a tumor more completely and deeply. This results in improved chemotherapy.

30 Alternatively, the RAIT therapy agent can be attached to bsAb rather the targetable conjugate. For example, an anti-CEA x anti-DTPA bsAb conjugated to Y-90-DOTA is administered first to a patient with CEA-expressing tumors. In this instance, advantage is taken of the selectivity of certain anti-chelate mabs

in that an anti-indium-DTPA antibody do not bind to a yttrium-DOTA chelate. After the Y-90-DOTA-anti-CEA x anti-indium-DTPA has maximized at the tumor and substantially cleared non-target tissue, a conjugate of indium-DTPA-glucuronidase is injected and localized specifically to the CEA tumor sites. The
5 patient is then injected with a polymer-prodrug such as poly(Glu)(SN-38)₁₀. The latter is cleaved selectively at the tumor to active monomeric SN-38, successfully combining chemotherapy with the previously administered RAIT.

It should also be noted that a bi-specific antibody or antibody fragment can be used in the present method, with at least one binding site specific to an
10 antigen at a target site and at least one other binding site specific to an enzyme. Such an antibody can bind the enzyme prior to injection, thereby obviating the need to covalently conjugate the enzyme to the antibody, or it can be injected and localized at the target site and, after non-targeted antibody has substantially
15 cleared from the circulatory system of the mammal, enzyme can be injected in an amount and by a route which enables a sufficient amount of the enzyme to reach the pre-targeted bsAb and bind to it to form an antibody-enzyme conjugate *in situ*.

In one embodiment of the invention, a clearing agent may be used which is given between doses of the bsAb and the targetable conjugate. The present
20 inventors have discovered that a clearing agent of novel mechanistic action may be used with the invention, namely a glycosylated anti-idiotypic Fab' fragment targeted against the disease targeting arm(s) of the bsAb. In this embodiment an anti-CEA (MN 14 Ab) x anti-peptide bsAb is given and allowed to accrete in disease targets to its maximum extent. To clear residual bsAb, an anti-idiotypic
25 Ab to MN-14, termed WI2, is given as a glycosylated Fab' fragment. The clearing agent binds to the bsAb in a monovalent manner, while its appended glycosyl residues direct the entire complex to the liver, where rapid metabolism takes place. Then the therapeutic which is associated with the targetable conjugate is given to the patient. The WI2 Ab to the MN-14 arm of the bsAb
30 has a high affinity and the clearance mechanism differs from other disclosed mechanisms (see Goodwin, *et al.*, *ibid*), as it does not involve cross-linking, because the WI2-Fab' is a monovalent moiety.

In accordance with yet another aspect of the present invention, the present invention provides a kit suitable for treating or identifying diseased tissues in a patient, comprising a bi-specific antibody or antibody fragment having at least one arm that specifically binds a targeted tissue and at least one other arm that specifically binds a targetable conjugate, a first targetable conjugate which comprises a carrier portion which comprises or bears at least one epitope recognizable by the at least one other arm of the bi-specific antibody or antibody fragment, and one or more conjugated therapeutic or diagnostic agents, or enzymes, and, optionally, a clearing composition useful for clearing non-localized antibodies and antibody fragments. When the first targetable conjugate comprises an enzyme, the kit may optionally contain a prodrug, when the enzyme is capable of converting the prodrug to a drug at the target site, a drug which is capable of being detoxified in the patient to form an intermediate of lower toxicity, when the enzyme is capable of reconvertng the detoxified intermediate to a toxic form, and, therefore, of increasing the toxicity of the drug at the target site, or a prodrug which is activated in the patient through natural processes and is subject to detoxification by conversion to an intermediate of lower toxicity, when the enzyme is capable of reconvertng the detoxified intermediate to a toxic form, and, therefore, of increasing the toxicity of the drug at the target site, or a second targetable conjugate which comprises a carrier portion which comprises or bears at least one epitope recognizable by the at least one other arm of the bi-specific antibody or antibody fragment, and a prodrug, when the enzyme is capable of converting the prodrug to a drug at the target site. Instruments which facilitate identifying or treating diseased tissue also can be included in the kit. Examples include, but are not limited to application devices, such as syringes. Solutions required for utilizing the disclosed invention for identifying or treating diseased tissue also can be included in the kit.

Examples

Example 1) Synthesis of a Peptide Antigen:

5 The peptide, Ac-Phe-Lys(Ac)-Tyr-Lys(Ac)-OH, is assembled using a resin for solid-phase synthesis and attaching the first residue (lysine) to the resin as the differentially protected derivative alpha-Fmoc-Lys(Aloc)-OH. The alpha-Fmoc protecting group is selectively removed and the Fmoc-Tyr(OBut), alpha-Fmoc-Lys(Aloc)-OH, and Fmoc-Phe-OH added with alternate cycles of coupling and alpha-amino group deprotection.

10 The Aloc - and OBut- side-chain protecting groups are then removed by reaction with TFA and the free alpha- and epsilon-amino groups are capped by reaction with acetic anhydride to give Ac-Phe-Lys(Ac)-Tyr-Lys(Ac)-OH.

15 Example 2) Coupling of Ac-Phe-Lys(Ac)-Tyr-Lys(Ac)-OH to KLH:

The peptide, Ac-Phe-Lys(Ac)-Tyr-Lys(Ac)-OH, dissolved in water and pH-adjusted to 4.0 with 1N HCl, is treated with a molar equivalent of 1-ethyl-3(3-dimethylaminopropyl) carbodiimide and allowed to react for 1 h at 4°C. Keyhole limpet hemocyanin (KLH) buffered at pH 8.5 is

20 treated with a 100-fold molar excess of the activated peptide and the conjugation reaction is allowed to proceed for 1 h at 4°C. The peptide-KLH conjugate is purified from unreacted peptide by size-exclusion chromatography and used for antibody production.

25 Example 3) Generation of an Anti-Peptide Ab:

Immunocompetent mice are injected with a mixture of the peptide antigen in complete Freund's adjuvant. Two booster shots of the peptide mixed with incomplete Freund's adjuvant are administered over the next several weeks. Spleen cells are harvested from the animals and fused with

30 Sp2/0-Ag14 myeloma cells. Culture supernatants of the resulting clones are analyzed for anti-peptide reactivity by ELISA, using plates coated with the original peptide immunogen. Enzyme-deficient hybridomas are

isolated to enable selection of fused cell lines, and selected clones grown in culture media to produce the anti-peptide Abs.

Example 4) Purification of Anti-Peptide Ab:

5 Anti-peptide Ab is purified chromatographically using a protein A column to isolate the IgG fraction, followed by ion-exchange columns to clean the desired product. The Ab of interest is finally purified by using an affinity column comprised of the peptide of interest bound to a solid support, prepared by chemically coupling said peptide to activated beads
10 or resin.

Example 5) Digestion of Anti-Peptide Ab to F(ab')₂:

The anti-peptide Ab is incubated with 200 µg/µL of pepsin at pH 4 for one hour and purified by a tandem column of protein A, to remove
15 undigested IgG, followed by G-50-Sephadex, to remove low molecular weight contaminants.

Example 6) Reduction of Anti-Peptide-Ab to Fab'-SH:

20 The anti-peptide-F(ab')₂ is reduced to a Fab' fragment by reaction with a freshly prepared cysteine solution in 0.1M PBS, containing 10mM EDTA. The progress of the reaction is followed by HPLC, and when complete, in about 1 h, the Fab'-SH is purified by spin-column chromatography and stored in deoxygenated buffer at pH < 5 containing
25 10mM EDTA.

Example 7) Oxidative Coupling of Anti-CEA-IgG to a Maleimide Moiety:

Anti-CEA Ab IgG is oxidized by reaction with 10mM sodium periodate for 90 minutes at 4°C, in the dark. The oxidized Ab is purified by spin-column chromatography and mixed with an excess of the cross-linker 4-
30 (4-maleimidophenyl) butyric acid hydrazide (MPBH). The reaction is allowed to proceed for 2 h and the IgG-hydrazone-maleimide purified by spin-column chromatography. The hydrazone bond is reduced by reaction with 10mM sodium cyanoborohydride and repurified.

Example 8) Preparation of anti-CEA-IgG x anti-Peptide-Fab' Bi-specific Ab:

5 The IgG-hydrazide-maleimide from Example 7) is treated with an equimolar amount of anti-peptide Fab'-SH, prepared in Example 6, at pH 6.0, for 30 minutes at room temperature. Remaining free thiol groups are blocked by a 30-minute reaction with iodoacetamide. The bi-specific Ab anti-CEA-IgG x anti-peptide-Fab' is purified by size-exclusion chromatography to remove unreacted Fab', followed by affinity chromatography using solid-phase-bound peptide to separate IgG x Fab' from unreacted IgG.

10

Example 9) Synthesis of Ac-Phe-Lys(Bz-DTPA)-Tyr-Lys(Bz-DTPA)-NH₂:

15 The peptide, Ac-Phe-Lys(DTPA)-Tyr-Lys(DTPA)-NH₂, is assembled using a resin for solid-phase synthesis and attaching the first residue (lysine to said resin as the differentially protected derivative alpha-Fmoc-Lys(Aloc)-OH. The alpha-Fmoc protecting group is selectively removed and the Fmoc-Tyr(OBut), alpha-Fmoc-Lys(Aloc)-OH, and Fmoc-Phe-OH added with alternate cycles of coupling and alpha-amino group deprotection. The Aloc- side-chain is removed by reaction with

20 palladium (0) catalyst. Alternatively, Boc-group protecting groups may be used which may be removed by reaction with TFA and the free amino groups reacted with excess of the ITC-Bz-DTPA. After removing excess Bz-DTPA, the alpha-amino group is capped by reaction with acetic anhydride, and the entire peptide removed from the resin with TFA (with

25 concomitant deprotection of the tyrosyl residue) to give Ac-Phe-Lys(Bz-DTPA)-Tyr-Lys(Bz-DTPA)-NH₂.

Example 10) Radiolabeling of Ac-Phe-Lys(Bz-DTPA)-Tyr-Lys(Bz-DTPA)-NH₂ with Y-90:

30 The title peptide in 100-fold molar excess is mixed with yttrium-90 radionuclide in acetate buffer at pH 5.5. The radiolabeling is complete and quantitative after 30 minutes.

Example 11) Conjugation of a Carboxylesterase to di-DTPA-Peptide:

Carboxylesterase (5 mg) in 0.2 M phosphate buffer, pH 8.0, is treated with a five-fold molar excess of the cross-linking agent sulfo-succinimidyl-[4-maleimidomethyl]-cyclohexane-1-carboxylate (sulfo-SMCC). After stirring two hours at room temperature, the activated enzyme is separated from low molecular weight contaminants using a spin-column of G-25 Sephadex and equilibrated in 0.1 M phosphate buffer, pH 7, containing 1 mM EDTA. The tetrapeptide N-acetyl-Cys.Lys(DTPA).Tyr.Lys(DTPA).NH₂ (ten-fold molar excess) is added to the activated enzyme and dissolved in the same buffer as used in the spin-column. After stirring for one hour at room temperature, the carboxylesterase-Cys.Lys(DTPA).Tyr.Lys(DTPA).NH₂ peptide conjugate is purified from unreacted peptide by spin-column chromatography on G-25 Sephadex in 0.25 M acetate buffer, pH 6.0. Successful conjugation is demonstrated by indium-111 labeling of an aliquot of the conjugate, and analysis by size-exclusion HPLC.

Example 12) Use of anti-CEA-IgG x anti-Peptide-Fab' Bi-specific Ab for

20 RAIT:

A patient with a CEA-expressing tumor burden is given anti-CEA-IgG x anti-peptide-Fab' bi-specific Ab. Seven days later, the patient is given Y-90-di-Bz-DTPA-peptide (from Example 10). The Y-90-labeled peptide clears rapidly from non-target tissue but localizes avidly to sites pre-targeted with the anti-CEA-IgG x anti-peptide-Fab' bi-specific Ab, effecting destruction of tumors.

Example 13) Preparation of a Galactose-WI2-Fab' Clearing Agent:

The anti-idiotypic Ab to MN-14, termed WI2 is digested to a F(ab')₂ fragment using pepsin, as outlined in Example 4. The F(ab')₂ is reduced to a Fab' fragment using a low molecular weight thiol, as outlined in Example 6. At the end of the reduction, the Fab'-SH is purified by spin-column chromatography and reacted with excess iodoacetamide to block

hinge-region thiol groups and prevent reassociation. After repurification from excess iodoacetamide the Fab' is reacted with a 400-fold molar excess of the galactosylation agent, the thio-imidate of cyanomethyl-2,3,4,6-tetra-O-acetyl-1-thio-beta-D-galactopyranoside (see Karacay et al.). The galactosylated protein is purified by two spin-columns and the galactose:Fab' ratio determined by MALDI-MS.

Example 14) Use of anti-CEA-IgG x anti-Peptide Fab' Bi-specific Ab for RAIT, with a bsAb Clearing Step:

A patient with a CEA-expressing tumor burden is given anti-CEA-IgG (MN-14) x anti-peptide-Fab' bi-specific Ab. Three days later, the patient is given a clearing dose of galactose-WI2-Fab'. Twenty-four hours after the clearing dose of a galactose-WI2-Fab', the patient is given Y-90-di-Bz-DTPA-peptide. The Y-90-labeled peptide clears rapidly from non-target tissue but localizes avidly to sites pretargeted with the anti-CEA-IgG x anti-peptide-Fab' bi-specific Ab, effecting destruction of tumors.

Example 15) Synthesis of Ac-Lys(DTPA)-TyrLys(DTPA)-Lys(TscG-Cys-)-NH₂ (IMP 192):

The first amino acid, Aloc-Lys(Fmoc)-OH was attached to 0.2 l mmol Rink amide resin on the peptide synthesizer followed by the addition of the Tc-99m ligand binding residues Fmoc-Cys(Trt)-OH and TscG to the side chain of the lysine using standard Fmoc automated synthesis protocols to form the following peptide: Aloc-Lys(TscG-Cys(Trt)-rink resin. The Aloc group was then removed by treatment of the resin with 8 mL of a solution containing 100 mg Pd[P(Ph)₃]₄ dissolved in 10 mL CH₂Cl₂, 0.75 mL glacial acetic acid and 2.5 ml diisopropylethyl amine. The resin mixture was then treated with 0.8 ml tributyltin hydride and vortex mixed for 60 min. The peptide synthesis was then continued on the synthesizer to make the following peptide: Lys(Aloc)-Tyr-Lys(Aloc)-Lys(TscG-Cys-)-rink resin. The N-terminus was acetylated by vortex mixing the resin for 60 mm with 8 mL of a solution containing 10 mL DMF, 3 mL acetic anhydride, and 6 mL diisopropylethylamine. The side

chain Aloc protecting groups were then removed as described above and the resin treated with piperidine using the standard Fmoc deprotection protocol to remove any acetic acid which may have remained on the resin. Activated DTPA and DTPA Addition: The DTPA, 5 g was dissolved in 40 mL 1.0 M tetrabutylammonium hydroxide in methanol. The methanol was removed under hi-vacuum to obtain a viscous oil. The oil was dissolved in 50 mL DMF and the volatile solvents were removed under hi-vacuum on the rotary evaporator. The DMF treatment was repeated two more times. The viscous oil was then dissolved in 50 mL DMF and mixed with 5 g HBTU. An 8 ml aliquot of the activated DTPA solution was then added to the resin which was vortex mixed for 14 hr. The DTPA treatment was repeated until the resin gave a negative test for amines using the Kaiser test. Cleavage and Purification: The peptide was then cleaved from the resin by treatment with 8 ml of a solution made from 30 ml TFA, 1 ml triisopropylsilane, and 1 ml ethanedithiol for 60 mm. The crude cleaved peptide was precipitated by pouring into 30 ml ether and was collected by centrifugation. The peptide was then purified by reverse phase HPLC using a 4 x 30 cm Waters preparative C-18 Delta-Pak column (15 μ m, 100Å). The HPLC fractions were collected and lyophilized to obtain a fraction which contained the desired product by ESMS ($MH \pm 1590$). Kit Formulation: The peptide was formulated into lyophilized kits which contained 78 μ g of the peptide, 0.92 mg non-radioactive $InCl_3$, 100 μ g stannous chloride, 3 mg gentisic acid, and HPCD (10 % on reconstitution).

Example 16) Tc-99m Labeling and Stability:

An IMP 192 kit was labeled by reconstituting the contents of the vial with 1.5 mL of saline which contained 25 mCi $Na^{99m}TcO_4$. The kit was incubated at room temperature for 10 mm and then heated in a boiling water bath for 15 mm. The labeled peptide solution was then cooled to room temperature. Aliquots were removed for stability studies. The aliquots were diluted 1:10 in saline, 1 mM cysteine in 0.05M phosphate pH 7.5, and fresh human serum. The original kit solution, the saline

- 5 dilution, and the cysteine challenge were incubated at room temperature while the serum sample was incubated at 37°C. The samples were monitored by HPLC and ITLC. The labeled peptide was stable in the *in vitro* tests. The retention time of the labeled peptide in serum was shifted from 6.3 min to 7.3 min. The shift may be due to ion pairing of some serum component with the peptide.

| Sample | Initial Label | First Time Point | Second Time Point | ITLC 24 hr Saturated NaCl |
|--|--|---|---|--------------------------------------|
| Kit Room Temp. | 1 % Void Vol 99 % Peptide (6.4 mm) | 3 hr 1 % Void Vol 99 % Peptide | 21 hr 5 % Void Vol 95 % Peptide | 5 % Solvent Front 94 % Origin |
| Saline Dilution Room Temp. | | 1.5 hr 1 % Void Vol 99 % Peptide | 19 hr 4 % Void Vol 96 % Peptide | 2.3 % Solvent Front 97 % Origin |
| Cys Challenge 1 mM in 0.05 M phosphate pH 7.5 Room Temp. | | 1 hr 2 % Void Vol 98 % Peptide | 19.5 hr 11 % Void Vol 89 % Peptide | 7.4 % Solvent Front 91.3 % Origin |
| Human Serum 37°C | | 2 hr 1 % Void Vol 7 % 6 min 92 % 7.2 min | 20 hr 3 % Void Vol 15 % 6 min 82 % 7.3 min | 1.7 % Solvent Front 96 % Origin |

10 **Example 17) Preparation of hMN-14 x 734 (Fab x Fab):**

- This bsMAb was prepared by crosslinking the hMN-14 Fab'_{SH} (a humanized monoclonal anti-CEA antibody) and 734 Fab'_{mal} (a murine anti-diDTPA) fragments, analogously to Example 8. The Fab'_{SH} fragments of hMN-14 and 734 were prepared by reduction of the F(ab')₂ fragments with 10 mM 2-mercaptoethylamine in the presence of 10 mM EDTA at pH 7.3 for 60 min at 37°C. Fab'_{SH} was collected after spin column (Penefsky) purification (Sephadex G-50-80, 50 mM NaOAc, 0.5 mM EDTA, pH 5.3) Maleimide group(s) were introduced onto 734 Fab'_{SH} fragment using 4 mM N,N'-o-phenylenedimaleimide at RT for 60 min. Spin column purification was used to isolate the Fab'_{mal}. Crosslinking of 734 Fab'_{mal} and hMN-14 Fab'_{SH} was allowed to proceed 16 h at 4°C at 1:1 molar ratio. To break the disulfide bonds which might have formed during this time, the reaction mixture was treated with 10

5 mM 2-mercaptoethylamine for 1h at pH 5.3 at 23°C. The SH groups were blocked with N-ethylmaleimide at pH 6.4. The reaction mixture was applied to a spin column to remove excess small molecular weight compounds. The bsAb was then isolated after purification on an analytical size exclusion HPLC column, Bio-Sil SEC-250. The HPLC retention time of the purified bsAb was 10.23 min.

Example 18) HPLC binding studies:

10 The bsMAb was radiolabeled using chloramine T (Greenwood and Hunter). Binding of the radioiodinated bsMAbs to CEA, WI2 (rat anti-MN-14 idiotype antibody) and radiolabeled peptidyl DTPA chelate was examined on analytical size exclusion HPLC. Approximately 90 % of the radioiodinated bsMAb bound to CEA upon treatment with 10-20x molar excess of CEA. The bsMAb complexed with radiolabeled indium-DTPA chelates (IMP-156 or IMP-192).

IMP 156 Ac-Phe-Lys(DTPA)-Tyr-Lys(DTPA)-NH₂

Example 19) Serum stability:

20 Radioiodinated bsMAb was tested for stability in fresh human serum at 37°C under a humidified 5 % CO₂ atmosphere. Aliquots were examined on SE-HPLC. In order to detect radioiodine associated with serum proteins, the aliquots were mixed with WI2 to shift the bsMAb peak to earlier retention times. The bsMAbs showed 3-5 % loss of binding capacity to WI2 after 48 h incubation in serum. Slight aggregate formation (4-7 %) was observed upon incubation of the bsMAbs in serum for 72 h.

Example 20) 99m-Tc-IMP-192:

30 *In vitro* stability of the Tc-99m complex of this peptidyl chelate was established by incubations in saline, fresh human serum and 10 mM cysteine for up to 20 h. *In vivo* stability was examined by analysis of urine collected from a mouse injected with 99m-Tc-IMP-192 in a

pretargeting experiment. The activity excreted in the urine appears to be the intact peptide because the activity still binds to the antibody as shown by SE-HPLC. Biodistribution studies of 99m-Tc-IMP-192 in normal BALB/c mice showed rapid blood clearance, Table 1. The *in vitro* and *in vivo* studies clearly demonstrate stability of 99m-Tc-IMP-192.

Table 1: Clearance of 99m-Tc-IMP-192 in BALB/c mice.

| Tissue | %ID/g | | | |
|-----------|-------------|-------------|-------------|-------------|
| | 1 h | 2 h | 4 h | 24 h |
| Liver | 0.27 ± 0.18 | 0.22 ± 0.16 | 0.09 ± 0.02 | 0.04 ± 0.0 |
| Spleen | 0.08 ± 0.01 | 0.09 ± 0.3 | 0.05 ± 0.02 | 0.03 ± 0.01 |
| Kidney | 4.16 ± 0.75 | 4.05 ± 0.60 | 3.21 ± 0.99 | 1.21 ± 0.08 |
| Lungs | 0.50 ± 0.23 | 0.29 ± 0.08 | 0.19 ± 0.04 | 0.05 ± 0.00 |
| Blood | 0.30 ± 0.09 | 0.21 ± 0.03 | 0.14 ± 0.04 | 0.05 ± 0.01 |
| Stomach | 0.39 ± 0.18 | 0.42 ± 0.18 | 0.27 ± 0.33 | 0.02 ± 0.01 |
| Small int | 1.37 ± 0.75 | 0.60 ± 0.06 | 0.21 ± 0.09 | 0.03 ± 0.01 |
| Lg.Int. | 0.41 ± 0.54 | 1.53 ± 0.45 | 1.58 ± 0.70 | 0.15 ± 0.14 |
| Muscle | 0.10 ± 0.06 | 0.05 ± 0.00 | 0.03 ± 0.01 | 0.00 ± 0.0 |
| Urine | 169 ± 95 | 57 ± 15 | 6.30 ± 4.53 | 0.20 ± 0.02 |

Example 21) Construction and expression of hMN14Fab-734scFv:

Recombinant methods were used to produce a monovalent bi-specific fusion protein comprising a Fab fragment derived from a humanized monoclonal anti-CEA antibody and a scFv derived from a murine anti-diDTPA. See Figure 3. The structure of single chain 734 (734scFv) was designed as GGGS-VL-(GGGGS)3-VH, in which the proximal GGGS provides a flexible linkage for the scFv to be connected to the constant region of the heavy chain of hMN-14 (Figure 1). Alternatively, the scFv can be connected to the constant region of the light chain of hMN-14. Appropriate linker sequences necessary for the in-frame connection of the hMN14 heavy chain Fd to 734scFv were introduced into the VL and VK domains of 734 by PCR reactions using specific primer sets. PCR-amplification of 734VL was performed using the primer set 734VLscFv5'(Cys) and 734VLscFv3' (respectively, SEQ ID NO's: 1 & 2). The primer 734VLscFv5'(Cys) represents the sense-

strand sequence encoding the first four residues (PKSC) of the human IgG1 hinge, linked in-frame to the first six residues (QLVVTQ) of 734 VL, via a short flexible linker, GGGS. One cysteine of the human hinge was included because it is required for the interchain disulfide linkage between the hMN14 heavy chain Fd-734scFv fusion and the hMN14 light chain. A Pst1 site was incorporated (underline) to facilitate ligation at the intronic sequence connecting the CH1 domain and the hinge. The primer 734VLscFv3' represents the anti-sense sequence encoding the last six residues (TKLKIL) of the 734 VL domain and a portion of the flexible linker sequence (GGGGSGGGG), which is fused in-frame downstream of the VL domain. Following PCR amplification, the amplified product (~400 bp) first was treated with T4 DNA polymerase to remove the extra "A" residue added to the termini during PCR-amplification and subsequently was digested with Pst1. The resultant product was a double-stranded DNA fragment with a Pst1 overhang and a blunt end. PCR amplification of 734VH was performed using the primer set 734VHscFv5' and 734VHscFv3'(Sac1). Primer 734VHscFv5' (SEQ ID NO: 3) represents the sense-strand sequence encoding the remaining part of the flexible linker sequence (SGGGGS) connecting the VL and VH sequences, and the first six residues (EVKLQE) of the 734 VH domain. Primer 734VHscFv3'(Sac1) (SEQ ID NO: 4) represents the anti-sense sequence encoding the last six residues (TVTVSS) of 734 VH. Also included is a translation stop codon (*). The restriction sites Eag1 (**bold**) and Sac1(underlined) were incorporated downstream of the stop codon to facilitate subcloning. Similarly, the PCR-amplified VH product of ~400 bp was first treated with T4 DNA polymerase to remove the extra "A" residues at the PCR product termini, and then digested with Sac1, resulting in a VH DNA fragment with a blunt end-sticky end configuration. A pBlueScript (Stratagene, La Jolla)-based staging vector (HC1kbpSK) containing a SacII fragment of the human IgG1 genomic sequence was constructed. The genomic SacII fragment contains a partial 5' intron, the human IgG1 CH1 domain, the intronic sequence connecting the CH1 to the hinge, the

hinge sequence, the intronic sequence connecting the hinge to the CH2 domain, and part of the CH2 domain. The segment containing the hinge and part of the CH2 domain in HC1kbpSK was removed by Pst1/Sac1 digestion, and the cloning site generated was used to co-ligate the VL (Pst1/blunt) and VH (blunt/Sac1) PCR products prepared above. The CH1 domain in the resultant construct (CH1-734pSK) is connected to the 734scFv gene sequence via an intron (Figure 4). Since the genomic SacII fragment for IgG1 only included part of the 5' intron sequence flanking the CH1 domain, the full intronic sequence was restored by inserting the remaining intronic sequence as a BamH1/SacII segment, into the corresponding sites of the CH1-734pSK. The BamH1/Eag1 fragment containing the full 5' intron, CH1 domain, connecting intron, 5 hinge-residues, short GGS linker, and a 734scFv sequences was then isolated, and used to replace the HindIII/Eag1 segment containing the human genomic IgG1 constant sequence in the hMN14pdHL2 vector. A HNB linker (SEQ ID NO: 5) with a BamH1 overhang on one end and a HindIII overhang on the other was used to facilitate the BamH1/Eag1 fragment ligation into the HindIII/Eag1 site in the hMN14pdHL2 vector. The resultant vector was designated hMN14-734pdHL2 and can be used to transfect mammalian cells for the expression of the bispecific protein. The hMN14pdHL2 vector was derived from the vector, pdHL2, which has previously been described. See Losman *et al.*, *Cancer Supplement*, 80:2660, 1997. Construction of hMN14pdHL2 was performed by replacing the VH and VK domains of hLL2pdHL2 with that of hMN14 using standard molecular biology techniques (Figure 5). The hMN14-734pdHL2 vector was transfected into SP2/0 cells by electroporation and the cell clones secreting bsAb were identified. The bsAb purified from cell culture supernatant (clone 341.1G6) on a protein L column (Pierce, Rockford, IL) is a 75 kD protein (based on amino acid sequence calculation) that co-migrated with the 66 kD marker in non-reducing SDS-PAGE probably due to secondary structure (Figure 2, lane 2). Under reducing conditions, bands corresponding to a heavy (50 kD) and a light (25 kD) chain were observed (Figure 2, lane 4). Kappa chain

monomers (25 kD) and dimers (50 kD) secreted by the transfectoma also were co-purified (Figure 2, lane 2) since protein L binds to kappa light chains of human, mouse and rat. Further separation of bsAb from kappa mono- and dimers is accomplished with ion-exchange chromatography. Purified hMN14Fab-734scFv shows specific binding to both CEA and In-DTPA-BSA in a dose dependent manner.

Example 22) Transgenic production of bscAb in milk:

A bscAb fragment is cloned into an expression vector containing a 5' casein promoter sequence and 3' untranslated genomic sequences that flank the insertion site. The expression cassette is then injected into the pronuclei of fertilized, mouse eggs, using procedures standard in the art. The eggs are then implanted into the uterus of a recipient female and allowed to gestate. After birth, the progeny are screened for the presence of the introduced DNA by Southern analysis. Milk from transgenic females is analyzed for the presence and functionality of the bscAb using standard immunological methods known in the art. The bscAb can be purified from the milk by complementary binding to an immobilized antigen, column chromatography or other methods known in the art.

Example 23) Transgenic production of bscAb in plants:

A bscAb fragment is cloned into an expression vector containing a shortened legumin B4 promoter plus 54 base pairs of LeB4 untranslated RNA leader from *Vicia faba* and encoding a LeB4 signal peptide, to direct the protein to the endoplasmic reticulum. The expression cassette is transformed into tobacco leaf discs according to the methods described by Zambryski *et al.*, using *Agrobacterium*-mediated gene transfer. Transformation is verified by Southern analysis. Transgenic plants are analyzed for the presence and functionality of the bscAb using standard immunological methods known in the art. The bscAb can be purified from the plant tissues using standard methods known in the art.

Example 24) Pretargeting Experiments:

Female nude mice (Taconic NCRNU, 3-4 weeks old) with GW 39 tumor xenografts were used for the pretargeting experiments. Tumors were 0.3-0.8 g.

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Table 2: Biodistribution of 125-I-hMN-14 x 734 bsAb and 111-In-indium-IMP-156 peptide in nude mice bearing GW-39 tumor xenografts:

hMN-14 x 734 was allowed 48 h for localization prior to 111-In-indium-IMP-156 injection. Biodistribution was performed 3 h post 111-In-indium-IMP-156. bsAb:peptide ratio administered, 1: 0.03. Five animals per time point.

| 15 | Tissue | 125-I-hMN-14 x 734 | | 111-In-indium-IMP-156 | |
|----|--------|--------------------|---------------|-----------------------|----------------|
| | | % ID/g | T/NT | % ID/g | T/NT |
| | tumor | 2.9 ± 1.1 | 1 | 5.2 ± 1.9 | 1 |
| | Liver | 0.1 ± 0.06 | 19 ± 6 | 0.5 ± 0.09 | 10.6 ± 3.5 |
| | Spleen | 0.5 ± 0.03 | 6.3 ± 1.2 | 0.5 ± 0.1 | 12 ± 6 |
| 20 | Kidney | 0.3 ± 0.08 | 9.3 ± 1.8 | 1.9 ± 0.5 | 2.6 ± 0.5 |
| | Lungs | 0.3 ± 0.1 | 12 ± 3 | 0.4 ± 0.1 | 12 ± 2 |
| | Blood | 0.3 ± 0.1 | 11 ± 2 | 0.7 ± 0.2 | 7.6 ± 1.5 |

25 Table 3: Control group showing the clearance of 111-In-indium-IMP-156 at 3 h after injection.

| 30 | Tissue | % ID/g | T/NT |
|----|--------|-----------------|----------------|
| | | | |
| | Tumor | 0.14 ± 0.02 | 1 |
| | Liver | 0.42 ± 0.1 | 0.3 ± 0.1 |
| | Spleen | 0.28 ± 0.09 | 0.5 ± 0.1 |
| | Kidney | 0.93 ± 0.13 | 0.2 ± 0.03 |
| | Lungs | 0.04 ± 0.01 | 3.5 ± 0.7 |
| 35 | Blood | 0.05 ± 0.01 | 3.1 ± 0.7 |

Table 4: Nude mice bearing GW 39 tumor xenografts were administered 125-I-labeled bsAb (5 μ Ci, 15 μ g, 1.5×10^{-10} mol). hMN-14 x 734 was allowed 24 h for localization and clearance before administering 99m-Tc-IMP-192 (10 μ Ci, 1.6×10^{-11} mol of peptide). Biodistribution studies were performed at 30 min, 1, 3 and 24 h post 99m-Tc-IMP-192 injection, five animals per time point. BsAb:peptide, 1: 0.1.

| 125-I-hMN-14 x 734 | | % ID/g | | | |
|--------------------|--------|----------------|----------------|----------------|----------------|
| 10 | Tissue | 30 min | 1 h | 3 h | 24 h |
| | Tumor | 4.9 \pm 1.1 | 6.0 \pm 2.3 | 5.5 \pm 1.1 | 3.3 \pm 0.7 |
| | Liver | 0.6 \pm 0.1 | 0.5 \pm 0.2 | 0.5 \pm 0.1 | 0.1 \pm 0.02 |
| | Spleen | 0.8 \pm 0.3 | 0.7 \pm 0.3 | 0.7 \pm 0.2 | 0.2 \pm 0.03 |
| 15 | Kidney | 0.5 \pm 0.1 | 0.5 \pm 0.1 | 0.5 \pm 0.1 | 0.1 \pm 0.02 |
| | Lungs | 0.9 \pm 0.3 | 0.8 \pm 0.2 | 0.8 \pm 0.3 | 0.3 \pm 0.1 |
| | Blood | 0.9 \pm 0.3 | 1.2 \pm 0.4 | 1.1 \pm 0.3 | 0.2 \pm 0.07 |
| 20 99m-Tc-IMP-192 | | % ID/g | | | |
| | Tissue | 30 min | 1 h | 3 h | 24 h |
| | Tumor | 11.4 \pm 4.8 | 14.3 \pm 3.6 | 12.6 \pm 5.2 | 8.7 \pm 3.3 |
| | Liver | 1.4 \pm 0.3 | 0.9 \pm 0.2 | 0.6 \pm 0.1 | 0.4 \pm 0.08 |
| 25 | Spleen | 1.2 \pm 0.4 | 0.8 \pm 0.2 | 0.5 \pm 0.1 | 0.4 \pm 0.2 |
| | Kidney | 9.9 \pm 6.1 | 4.6 \pm 0.7 | 2.4 \pm 0.5 | 1.2 \pm 0.3 |
| | Lungs | 4.2 \pm 3.4 | 3.6 \pm 1.9 | 1.0 \pm 0.3 | 0.3 \pm 0.1 |
| | Blood | 4.3 \pm 1.2 | 3.5 \pm 0.9 | 1.7 \pm 0.4 | 0.6 \pm 0.2 |

Table 5: Nude mice bearing GW 39 tumor xenografts were administered 125-I-labeled bsAb (5 μ Ci, 15 μ g, 1.5×10^{-10} mol). hMN-14 x 734 was allowed 24 h for localization and clearance before administering 99m-Tc-IMP-192 (10 μ Ci, 1.6×10^{-11} mol of peptide). Biodistribution studies were performed at 30 min, 1, 3 and 24 h post 99m-Tc-IMP-192 injection, five animals per time point. BsAb:peptide, 1: 0.1.

| 125-I-hMN-14 x | | Tumor / non-tumor ratio | | | |
|----------------|----------------|-------------------------|----------------|-----------------|--|
| Tissue | 30 min | 1 h | 3 h | 24 h | |
| Liver | 8.8 ± 1.5 | 12.1 ± 5.5 | 10.3 ± 2.5 | 23.8 ± 3.5 | |
| Spleen | 6.4 ± 1.6 | 9.3 ± 4.0 | 7.9 ± 1.7 | 18.2 ± 4.0 | |
| Kidney | 10.0 ± 2.6 | 12.5 ± 4.5 | 11.1 ± 3.0 | 27.3 ± 4.6 | |
| Lungs | 6.2 ± 2.3 | 8.4 ± 4.6 | 7.2 ± 2.3 | 12.4 ± 6.6 | |
| Blood | 5.7 ± 2.1 | 4.9 ± 1.2 | 5.1 ± 1.3 | 14.5 ± 3.6 | |
| 99m-Tc-IMP-192 | | Tumor / non-tumor ratio | | | |
| Tissue | 30 min | 1 h | 3 h | 24 h | |
| Liver | 7.9 ± 1.7 | 15.7 ± 5.4 | 20.7 ± 7.6 | 22.3 ± 7.4 | |
| Spleen | 9.4 ± 1.0 | 19.5 ± 8.6 | 22.9 ± 7.5 | 23.8 ± 3.5 | |
| Kidney | 1.2 ± 0.2 | 3.1 ± 0.6 | 5.2 ± 1.5 | 7.3 ± 1.9 | |
| Lungs | 3.7 ± 1.7 | 5.5 ± 3.6 | 13.5 ± 7.1 | 30.8 ± 14.4 | |
| Blood | 2.7 ± 0.7 | 4.2 ± 1.3 | 7.3 ± 2.3 | 16.1 ± 6.4 | |

Table 6: Control group of nude mice bearing GW-39 tumors received 99m-Tc-IMP-192 (10 μ Ci, 1.6×10^{-11} mol of peptide) and were sacrificed 3 h later.

| 99m-Tc-IMP-192 | |
|----------------|----------------|
| Tissue | % ID/g |
| Tumor | 0.2 ± 0.05 |
| Liver | 0.3 ± 0.07 |
| Spleen | 0.1 ± 0.05 |
| Kidney | 2.6 ± 0.9 |
| Lungs | 0.2 ± 0.07 |
| Blood | 0.2 ± 0.09 |

The percentage of the available DTPA binding sites on the tumor bound bsAb filled with 99m-Tc-IMP-192 was calculated from the above data assuming one peptide bound to one bsAb molecule. However, it is possible that one peptide molecule can crosslink two molecules of bsAb.

5

Table 7:

| | time | % saturation on hMN-14 x 734 |
|----|--------|---------------------------------|
| 10 | 30 min | 25.4 |
| | 1 h | 25.8 |
| | 3 h | 25 |
| | 24 h | 28 |

15

The foregoing experimental data show that: the humanized x murine bsAb retained its binding capability to CEA and indium-DTPA; the hMN-14 x 734 (Fab x Fab) effectively targets a tumor; the dual functional peptidyl Tc-99m chelator was stable; 99m-Tc-IMP-192 complexed to tumor-localized hMN-14 x 734 and was retained for at least 24 h; and imaging of tumors is possible at early time points (1-3h) post 99m-Tc-IMP-192 injection.

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Example 25) Use of anti-CEA Fab x anti-peptide scFv fusion protein for

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RAIT, with a bsAb Clearing Step:

A patient with a CEA-expressing tumor burden is given an anti-CEA Fab x anti-peptide scFv fusion protein. Three days later, the patient is given a clearing dose of galactose-WI2-Fab'. Twenty-four hours after the clearing dose of a galactose-WI2-Fab', the patient is given Y-90-di-Bz-DTPA-peptide. The Y-90-labeled peptide clears rapidly from non-target tissue but localizes avidly to sites pretargeted with the anti-CEA Fab x anti-peptide scFv fusion protein, effecting destruction of tumors.

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Example 26) Use of anti-CEA-IgG x anti-peptide Fab' Bi-specific Ab for

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Prodrug Therapy with a bsAb Clearing Step:

A patient with colorectal cancer is given an injection of an IgG-hMN-14 x anti-peptide Fab' bsAb. After 48 h, to allow for maximum accretion in

tumors, the patient is given a clearing dose of galactose-W12-Fab'. This amount is between 5 and 15 times the amount of primary bsAb remaining in circulation at the time-point specified. Three hours after administration of the galactose-W12-Fab', a tumor-saturating amount of the carboxylesterase-Cys.Lys(DTPA).Tyr.Lys(DTPA).NH₂ conjugate from example 11 is given, and allowed to clear circulation and normal tissues. After an additional three hours, a standard chemotherapy dose of CPT- 11 is administered to the patient. This protocol effectively generates free SN-38 specifically at the tumor target sites and effects the destruction of tumor cells.

Example 27) Preparation of a carboxylesterase-DTPA conjugate

Two vials of rabbit liver carboxylesterase (SIGMA; protein content ~ 17 mg) are reconstituted in 2.2 ml of 0.1 M sodium phosphate buffer, pH 7.7 and mixed with a 25-fold molar excess of CA-DTPA using a freshly prepared stock solution (~ 25 mg/ml) of the latter in DMSO. The final concentration of DMSO in the conjugation mixture is 3 % (v/v). After 1 hour of incubation, the mixture is pre-purified on two 5-mL spin-columns (Sephadex G50/80 in 0.1 M sodium phosphate pH 7.3) to remove excess reagent and DMSO. The eluate is purified on a TSK 3000G Supelco column using 0.2 M sodium phosphate pH 6.8 at 4 ml/min. The fraction containing conjugate is concentrated on a Centricon-10™ concentrator, and buffer-exchanged with 0.1 M sodium acetate pH 6.5. Recovery: 0.9 ml, 4.11 mg/ml (3.7 mg). Analytical HPLC analysis using standard conditions, with in-line UV detection, revealed a major peak with a retention time of 9.3 min and a minor peak at 10.8 min in 95-to-5 ratio. Enzymatic analysis showed 115 enzyme units/mg protein, comparable to unmodified carboxylesterase. Mass spectral analyses (MALDI mode) of both unmodified and DTPA-modified CE shows an average DTPA substitution ratio near 1.5. A metal-binding assay using a known excess of indium spiked with radioactive indium confirmed the DTPA:enzyme ratio to be 1.24 and 1.41 in duplicate experiments. Carboxylesterase-DTPA is labeled with

In-111 acetate at a specific activity of 12.0 mCi/mg, then treated with excess of non-radioactive indium acetate, and finally treated with 10 mM EDTA to scavenge off excess non-radioactive indium. Incorporation by HPLC and ITLC analyses is 97.7%. A HPLC sample is completely
5 complexed with a 20-fold molar excess of bispecific antibody hMN-14 Fab' x 734 Fab', and the resultant product further complexes with WI2 (anti-ID to hMN-14), with the latter in 80-fold molar excess with respect to bispecific antibody.

10 Combinations of the bi-specific constructs described in the present invention or others of similar specificities are suitable for pretargeted RAIT, where IMP-192 peptide and its analogues are labeled with therapeutic radioisotopes such as 188-Re, 213-Bi, 67-Cu and the like. It will be recognized that therapeutic chelates can be conjugated to peptides that have other than
15 chelate epitopes for recognition by bsAbs, as described above.

It will be appreciated as well that detectable radiolabels can be directed to a site of interest, e.g. a tumor, which is to be excised or otherwise detected and /or treated in intraoperative, endoscopic, intravascular or other similar
20 procedures, using the pretargeting methods of the present invention, in combination with various linkers. The pretargeting is effected with non-radioactive bsAbs and the eventual administration and localization of the low molecular weight radiolabeled linker, and clearance of unbound linker, are both comparatively rapid, compatible with surgical procedures that should avoid
needless delay and which can use radioisotopes with short half-lives.

25 Additionally, the disclosed therapies can be used for post-surgical radioimmunotherapy protocols to ensure the eradication of residual tumor cells.

All references cited herein are hereby incorporated herein by reference in their entireties.

30

Additional references of interest include the following:

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WHAT IS CLAIMED IS:

1. A method of treating or identifying diseased tissues in a patient, comprising:

(A) administering to said patient a bi-specific antibody or antibody fragment having at least one arm that specifically binds a targeted tissue and at least one other arm that specifically binds a targetable conjugate;

(B) optionally, administering to said patient a clearing composition, and allowing said composition to clear non-localized antibodies or antibody fragments from circulation;

(C) administering to said patient a first targetable conjugate which comprises a carrier portion which comprises or bears at least one epitope recognizable by said at least one other arm of said bi-specific antibody or antibody fragment, and one or more conjugated therapeutic or diagnostic agents, or enzymes; and

(D) when said targetable conjugate comprises an enzyme, further administering to said patient

1) a prodrug, when said enzyme is capable of converting said prodrug to a drug at the target site; or

2) a drug which is capable of being detoxified in said patient to form an intermediate of lower toxicity, when said enzyme is capable of reconvertng said detoxified intermediate to a toxic form, and, therefore, of increasing the toxicity of said drug at the target site, or

3) a prodrug which is activated in said patient through natural processes and is subject to detoxification by conversion to an intermediate of lower toxicity, when said enzyme is capable of reconvertng said detoxified intermediate to a toxic form, and, therefore, of increasing the toxicity of said drug at the target site, or

4) a second targetable conjugate which comprises a carrier portion which comprises or bears at least one epitope recognizable by said at least one other arm of said bi-specific

antibody or antibody fragment, and a prodrug, when said enzyme is capable of converting said prodrug to a drug at the target site.

2. The method of claim 1, further comprising, when said first targetable conjugate comprises a prodrug, administering a second targetable conjugate which comprises a carrier portion which comprises or bears at least one epitope recognizable by said at least one other arm of said bi-specific antibody or antibody fragment, and an enzyme capable of converting said prodrug to a drug or of reconverting a detoxified intermediate of said drug to a toxic form.

3. The method of claim 1, wherein said therapeutic agent comprises one or more radioactive isotopes useful for killing diseased tissue.

4. The method of claim 1, wherein said therapeutic agent comprises boron atoms, and said method further comprises the step of irradiating said boron atoms localized at said diseased tissue, thereby effecting BNCT of said diseased tissue.

5. The method of claim 4, wherein said boron atoms are boron-10 enriched.

6. The method of claim 1, wherein said therapeutic agent comprises one or more toxins.

7. The method of claim 1, wherein said therapeutic agent comprises one or more drugs.

8. The method of claim 1, wherein said therapeutic agent comprises one or more prodrugs.

9. The method of claim 1, wherein said diagnostic agent comprises one or more radioactive isotopes useful for detecting diseased tissue.

10. The method of claim 1, wherein said diagnostic agent comprises one or more image enhancing agents for use in magnetic resonance imaging (MRI).
11. The method of claim 9, wherein said radioactive isotope is used to perform positron-emission tomography (PET).
12. The method of claim 1, wherein said at least one arm that specifically binds a targeted tissue is a monoclonal antibody or a fragment of a monoclonal antibody.
13. The method of claim 1, wherein said at least one other arm that specifically binds a targetable conjugate is a monoclonal antibody or a fragment of a monoclonal antibody.
14. The method of claim 1, wherein said at least one arm that specifically binds a targeted tissue is a humanized antibody or a fragment of a humanized antibody.
15. The method of claim 1, wherein said at least one other arm that specifically binds a targetable conjugate is a humanized antibody or a fragment of a humanized antibody.
16. The method of claim 1, wherein said targetable conjugate comprises a peptide.
17. The method of claim 1, wherein said targetable conjugate comprises a carbohydrate.
18. The method of claim 1, wherein said targetable conjugate comprises one or more haptens.

19. The method of claim 1, wherein said targetable conjugate comprises one or more chelators or metal-chelate complexes.
20. The method of claim 1, wherein said bi-specific antibody or antibody fragment further comprises a radionuclide.
21. The method of claim 16, wherein said peptide is X-Gly-D-Tyr-D-Trp-Gly-D-Lys(X)-Gly-D-Tyr-D-Trp-OH, where X represents a moiety selected from the group consisting of a free amino acid group, a protected amino acid group, a chelating agent and a metal-chelate complex.
22. The method of claim 16, wherein said peptide is Ac-Cys(Y)-D-Tyr-D-Trp-Gly-D-Cys(Y)-Gly-D-Tyr-D-Trp-OH, where Y represents a moiety selected from the group consisting of a free thiol group, a protected thiol group, a chelating agent and a metal-chelate complex.
23. The method of claim 16, wherein said peptide is Ac-Gly-D-iodo-Tyr-D-Trp-Gly-D-Lys(Ac)-Gly-D-iodo-Tyr-D-Trp-OH.
24. The method of claim 16, wherein said peptide comprises two or more different chelators which can be complexed selectively with different metal ions, one or both of said cations being a diagnostically useful radioisotope or a therapeutic radioisotope.
25. The method of claim 19, wherein at least one of said chelators is a hard base chelator for a hard acid cation, and at least one of said chelators is a soft base chelator for a soft acid cation.
26. The method of claim 25, wherein said hard base chelator comprises carboxylate and amine groups.

27. The method of claim 26, wherein said hard base chelator is DTPA, NOTA, DOTA or TETA.

28. The method of claim 19, wherein said soft base chelator comprises at least one sulfur-containing group.

29. The method of claim 28, wherein said soft base chelator is a substituted or unsubstituted thiosemicarbazonylglyoxylcysteine (TscGCys) or thiosemicarbazinylacetylcysteine (TscACys).

30. A kit useful for treating or identifying diseased tissues in a patient comprising:

(A) a bi-specific antibody or antibody fragment having at least one arm that specifically binds a targeted tissue and at least one other arm that specifically binds a targetable conjugate;

(B) a first targetable conjugate which comprises a carrier portion which comprises or bears at least one epitope recognizable by said at least one other arm of said bi-specific antibody or antibody fragment, and one or more conjugated therapeutic or diagnostic agents, or enzymes; and

(C) optionally, a clearing composition useful for clearing non-localized antibodies and antibody fragments; and

(D) optionally, when said first targetable conjugate comprises an enzyme,

1) a prodrug, when said enzyme is capable of converting said prodrug to a drug at the target site; or

2) a drug which is capable of being detoxified in said patient to form an intermediate of lower toxicity, when said enzyme is capable of reconverting said detoxified intermediate to a toxic form, and, therefore, of increasing the toxicity of said drug at the target site, or

3) a prodrug which is activated in said patient through natural processes and is subject to detoxification by conversion to an intermediate of lower toxicity, when said enzyme is capable of

reconverting said detoxified intermediate to a toxic form, and, therefore, of increasing the toxicity of said drug at the target site, or

4) a second targetable conjugate which comprises a carrier portion which comprises or bears at least one epitope recognizable by said at least one other arm of said bi-specific antibody or antibody fragment, and a prodrug, when said enzyme is capable of converting said prodrug to a drug at the target site.

31. The kit of claim 30, further comprising, when said first targetable conjugate comprises a prodrug, a second targetable conjugate which comprises a carrier portion which comprises or bears at least one epitope recognizable by said at least one other arm of said bi-specific antibody or antibody fragment, and an enzyme capable of converting said prodrug to a drug or of reconverting a detoxified intermediate of said drug to a toxic form.

32. A bi-specific antibody or antibody fragment having at least one arm that specifically binds a targeted tissue and at least one other arm that specifically binds a targetable conjugate.

33. The bi-specific antibody or antibody fragment of claim 32, wherein said antibody or antibody fragment is humanized.

34. The bi-specific antibody or antibody fragment of claim 32, wherein said antibody or antibody fragment is monoclonal.

35. A recombinant DNA construct comprising an expression cassette capable of producing in a host cell a bi-specific antibody or antibody fragment having at least one arm that specifically binds a targeted tissue and at least one other arm that specifically binds a targetable conjugate, wherein said construct comprises, in the 5' to 3' direction of transcription, a transcriptional initiation regulatory region functional in said host cell, a translational initiation regulatory region functional in said host cell, a DNA sequence encoding said bi-specific

antibody or antibody fragment, and a transcriptional and translational termination regulatory region functional in said host cell, wherein said bi-specific antibody or antibody fragment is under the control of said regulatory regions.

36. A set of expression cassettes capable of producing a bi-specific antibody or antibody fragment having at least one arm that specifically binds to a targeted tissue and at least one other arm that specifically binds to a targetable conjugate, wherein each cassette comprises, in the 5' to 3' direction of transcription, a transcriptional initiation regulatory region functional in a host cell, a translational initiation regulatory region functional in said host cell, a DNA sequence encoding a fragment of said bi-specific antibody, and a transcriptional and translational termination regulatory region functional in said host cell, wherein said fragment is under the control of said regulatory regions.

37. A method of preparing a bi-specific antibody or antibody fragment having at least one arm that specifically binds a targeted tissue and at least one other arm that specifically binds a targetable conjugate, comprising:

- (A) introducing the recombinant DNA construct of claim 1 into a host cell;
- (B) growing said cell and isolating said antibody or antibody fragment.

38. The method of claim 37, wherein said host cell is a mammalian cell.

39. The method of claim 38, wherein said mammalian cell is a zygote, and said introduction of said recombinant DNA construct produces a transgenic animal capable of producing said bi-specific antibody or antibody fragment.

40. The method of claim 37, wherein said host cell is an insect cell.

41. The method of claim 37, wherein said host cell is an bacterial cell.

42. The method of claim 37, wherein one or more arms of said bi-specific antibody or antibody fragment has multiple binding sites for its respective hapten.

43. A method of preparing a bi-specific fusion protein having at least one arm that specifically binds a targeted tissue and at least one other arm that specifically binds a targetable conjugate, comprising:

- (1) (A) introducing into a host cell a recombinant DNA construct comprising an expression cassette capable of producing in said host cell a fragment of said bi-specific fusion protein, wherein said construct comprises, in the 5' to 3' direction of transcription, a transcriptional initiation regulatory region functional in said host cell, a translational initiation regulatory region functional in said host cell, a DNA sequence encoding a scFv linked to a Fd fragment, and a transcriptional and translational termination regulatory region functional in said host cell, wherein said fragment of said bi-specific fusion protein is under the control of said regulatory regions;
- (B) co-introducing into said host cell a recombinant DNA construct comprising an expression cassette capable of producing in said host cell a light-chain antibody fragment which is complementary to said Fd fragment in (A) and which when associated with said Fd fragment forms a Fab fragment whose binding site is specific for said targeted tissue, wherein said construct comprises, in the 5' to 3' direction of transcription, a transcriptional initiation regulatory region functional in said host cell, a translational initiation regulatory region functional in said host cell, a DNA sequence encoding a light-chain antibody fragment, and a transcriptional and translational termination regulatory region functional in said host cell, wherein said light-

chain antibody fragment is under the control of said regulatory regions;

(C) growing said cell and isolating said bi-specific fusion protein, or

(2) (A) introducing into a first host cell a recombinant DNA construct comprising an expression cassette capable of producing in said first host cell a fragment of said bi-specific fusion protein, wherein said construct comprises, in the 5' to 3' direction of transcription, a transcriptional initiation regulatory region functional in said first host cell, a translational initiation regulatory region functional in said first host cell, a DNA sequence encoding a scFv linked to a Fd fragment, and a transcriptional and translational termination regulatory region functional in said first host cell, wherein said fragment of said bi-specific fusion protein is under the control of said regulatory regions;

(B) introducing into a second host cell a recombinant DNA construct comprising an expression cassette capable of producing in said second host cell a light-chain antibody fragment which is complementary to said Fd fragment in (2)(A) and which when associated with said Fd fragment forms a Fab fragment whose binding site is specific for said targeted tissue, wherein said construct comprises, in the 5' to 3' direction of transcription, a transcriptional initiation regulatory region functional in said second host cell, a translational initiation regulatory region functional in said second host cell, a DNA sequence encoding a light-chain antibody fragment, and a transcriptional and translational termination regulatory region functional in said second host cell, wherein said light-chain antibody fragment is under the control of said regulatory regions;

(C) growing said first and second host cells;

(D) optionally isolating said bi-specific fusion protein fragment and said light-chain antibody fragment; and

(E) combining said fragments to produce a bi-specific fusion protein and isolating said bi-specific fusion protein.

44. A method of preparing a bi-specific fusion protein having at least one arm that specifically binds a targeted tissue and at least one other arm that specifically binds a targetable conjugate, comprising:

(1) (A) introducing into a host cell a recombinant DNA construct comprising an expression cassette capable of producing in said host cell a fragment of said bi-specific fusion protein, wherein said construct comprises, in the 5' to 3' direction of transcription, a transcriptional initiation regulatory region functional in said host cell, a translational initiation regulatory region functional in said host cell, a DNA sequence encoding a scFv linked to a light-chain antibody fragment, and a transcriptional and translational termination regulatory region functional in said host cell, wherein said fragment of said bi-specific fusion protein is under the control of said regulatory regions;

(B) co-introducing into said host cell a recombinant DNA construct comprising an expression cassette capable of producing in said host cell a Fd fragment which is complementary to said light-chain antibody fragment in (A) and which when associated with said light-chain antibody fragment forms a Fab fragment whose binding site is specific for said targeted tissue, wherein said construct comprises, in the 5' to 3' direction of transcription, a transcriptional initiation regulatory region functional in said host cell, a translational initiation regulatory region functional in said host cell, a DNA sequence encoding a Fd fragment, and a transcriptional and translational termination regulatory region functional in said host cell, wherein said Fd fragment is under the control of said regulatory regions;

(C) growing said cell and isolating said bi-specific fusion protein, or

(2) (A) introducing into a first host cell a recombinant DNA

construct comprising an expression cassette capable of producing in said first host cell a fragment of said bi-specific fusion protein, wherein said construct comprises, in the 5' to 3' direction of transcription, a transcriptional initiation regulatory region functional in said first host cell, a translational initiation regulatory region functional in said first host cell, a DNA sequence encoding a scFv linked to a light-chain antibody fragment, and a transcriptional and translational termination regulatory region functional in said first host cell, wherein said fragment of said bi-specific fusion protein is under the control of said regulatory regions;

(B) introducing into a second host cell a recombinant DNA construct comprising an expression cassette capable of producing in said second host cell a Fd fragment which is complementary to said light-chain antibody fragment in (2)(A) and which when associated with said light-chain antibody fragment forms a Fab fragment whose binding site is specific for said targeted tissue, wherein said construct comprises, in the 5' to 3' direction of transcription, a transcriptional initiation regulatory region functional in said second host cell, a translational initiation regulatory region functional in said second host cell, a DNA sequence encoding a Fd fragment, and a transcriptional and translational termination regulatory region functional in said second host cell, wherein said Fd fragment is under the control of said regulatory regions;

(C) growing said first and second host cells;

(D) optionally isolating said bi-specific fusion protein fragment and said Fd fragment; and

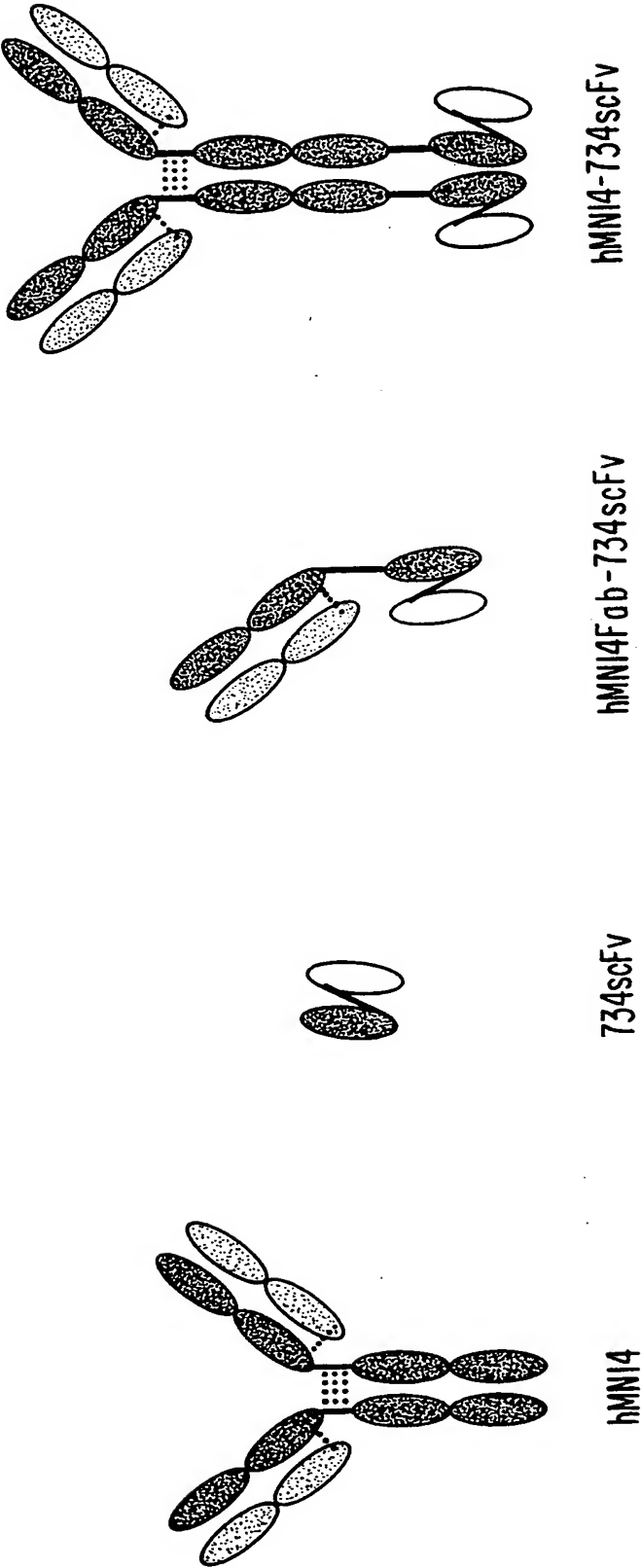
(E) combining said fragments to produce a bi-specific fusion protein and isolating said bi-specific fusion protein.

45. The method of claim 43, wherein said host cell is a mammalian cell.

46. The method of claim 43, wherein said host cell is an insect cell.
47. The method of claim 43, wherein said host cell is a bacterial cell.
48. The method of claim 44, wherein said host cell is a mammalian cell.
49. The method of claim 44, wherein said host cell is an insect cell.
50. The method of claim 44, wherein said host cell is a bacterial cell.

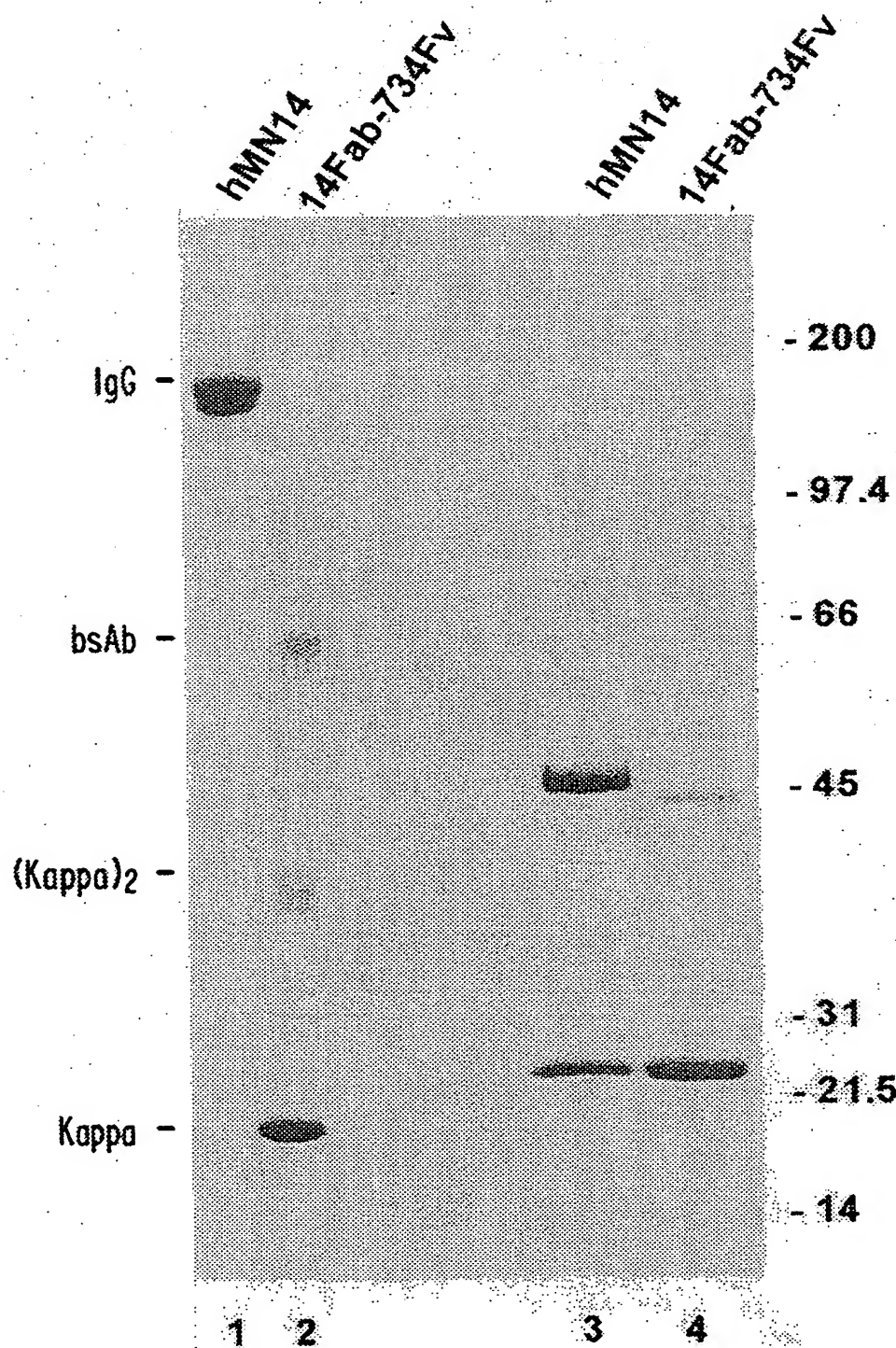
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FIG. 1



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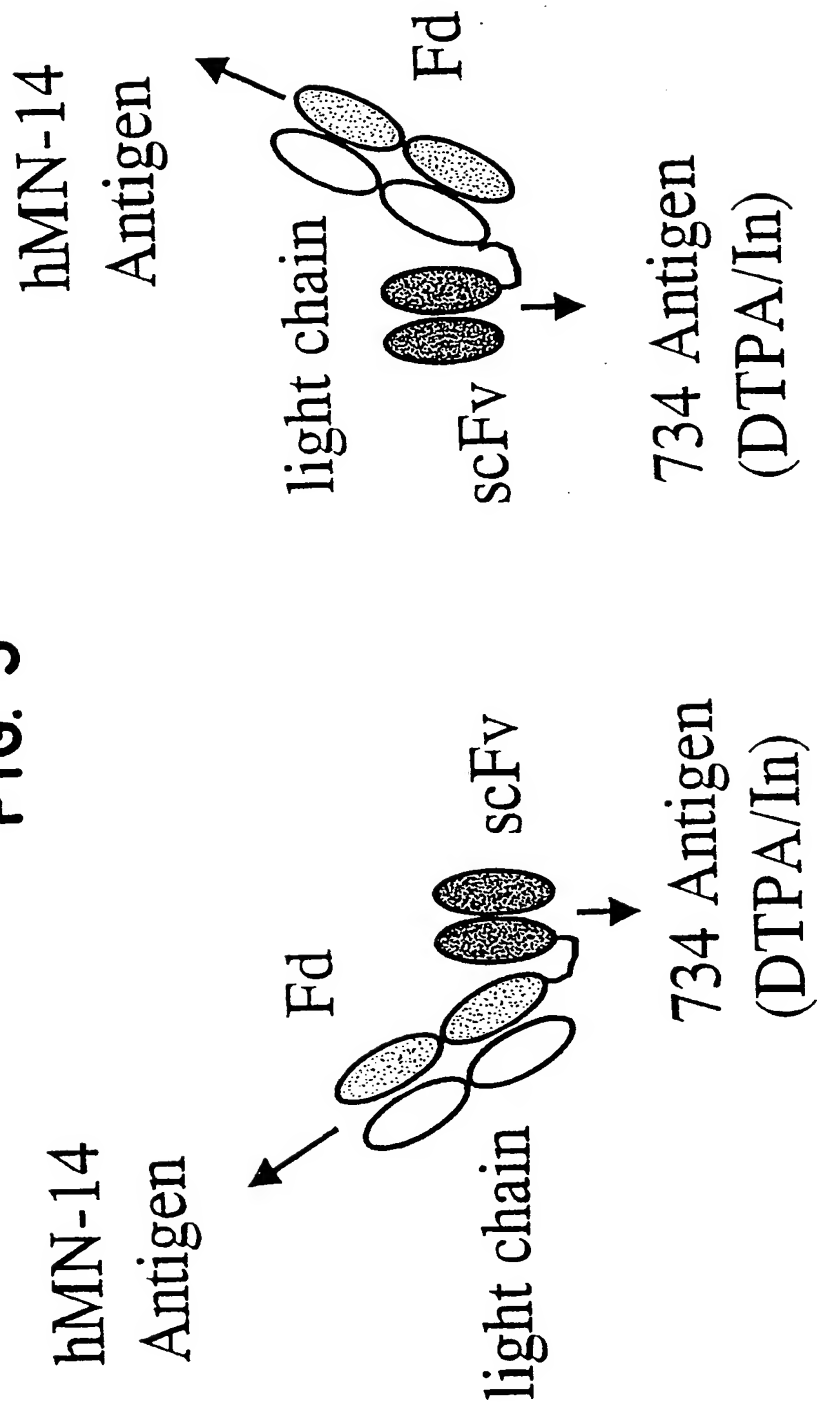
FIG. 2



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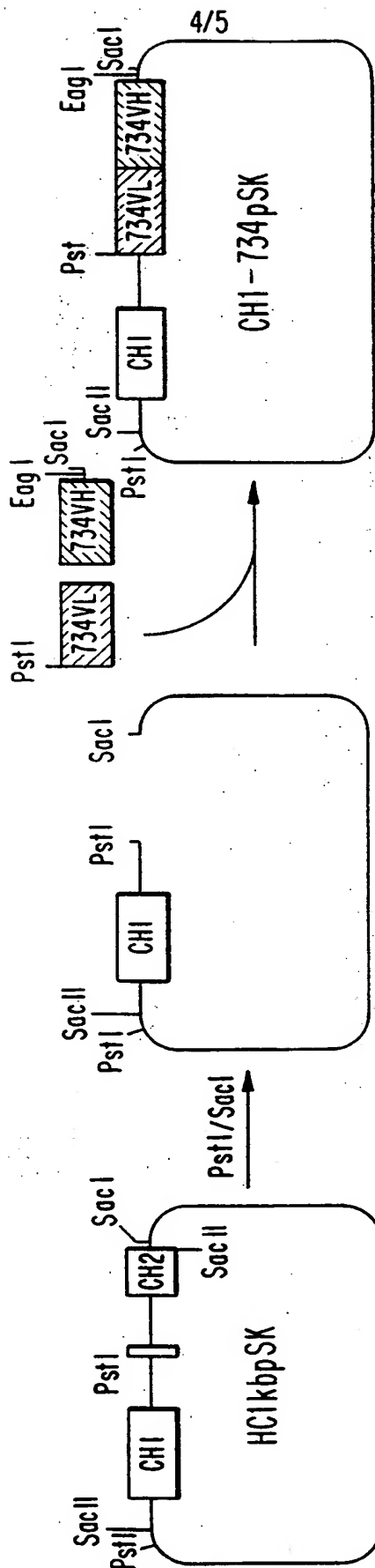
FIG. 3

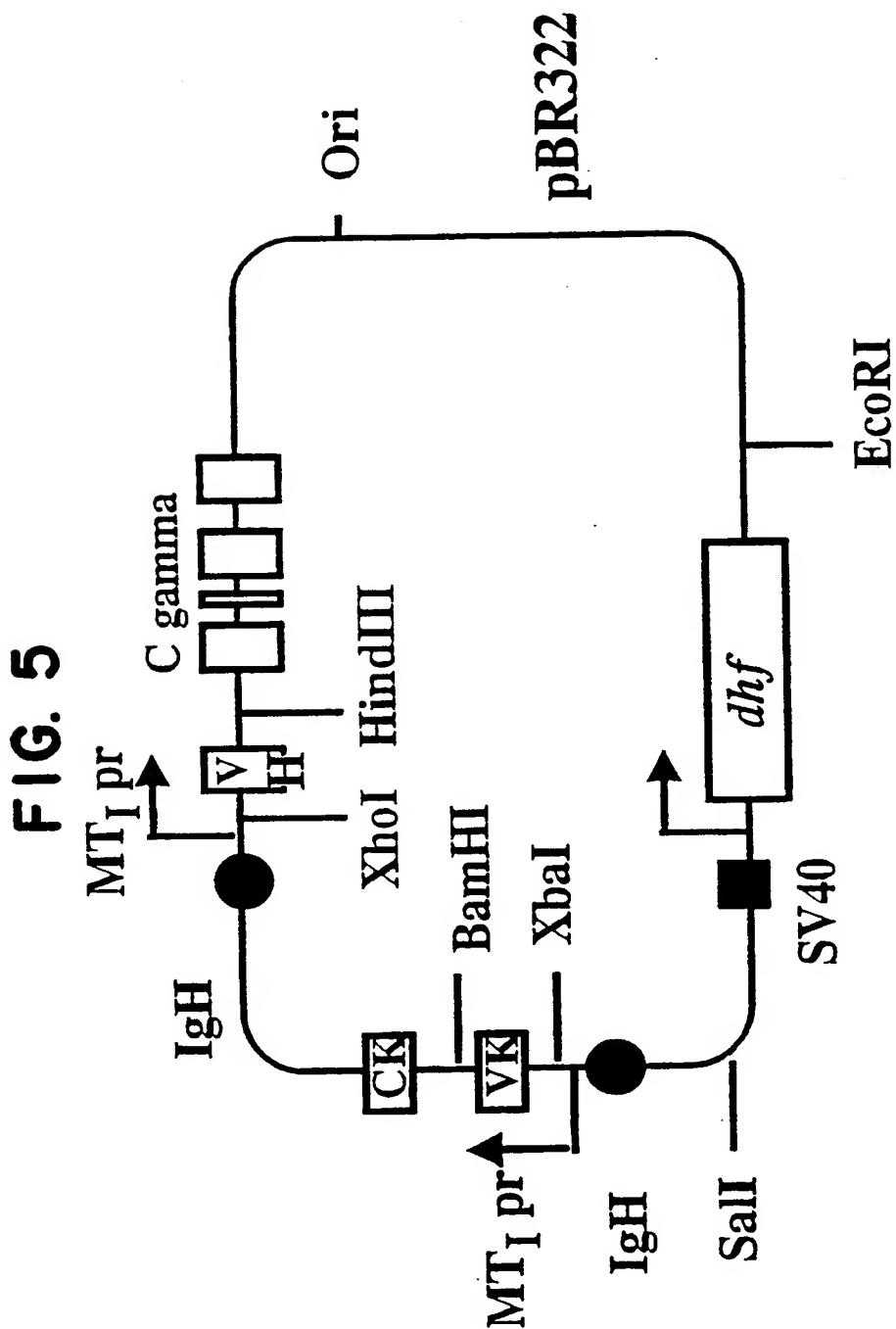


Bi-specific Construct B

Bi-specific Construct A

FIG. 4





Plasmid Map of MN14pdHL2

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| (21) International Application Number: PCT/US99/13879 (22) International Filing Date: 22 June 1999 (22.06.99) (30) Priority Data: 60/090,142 22 June 1998 (22.06.98) US 60/104,156 14 October 1998 (14.10.98) US (63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Applications US 60/090,142 (CIP) Filed on 22 June 1998 (22.06.98) US 60/104,156 (CIP) Filed on 14 October 1998 (14.10.98) (71) Applicant (for all designated States except US): IMMUNOMEDICS, INC. [US/US]; 300 American Road, Morris Plains, NJ 07950 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): HANSEN, Hans, J. [US/US]; 118 Moonraker Drive, Slidell, LA 70458 (US). GRIFFITHS, Gary, L. [GB/US]; 36 Edgehill Avenue, Morristown, NJ 07960 (US). LEUNG, Shui-on [-/US]; 31 Alexandria Road, Morris Township, NJ 07960 (US). | | (74) Agents: SAXE, Bernhard, D. et al.; Foley & Lardner, Suite 500, 3000 K Street, N.W., Washington, DC 20007-5109 (US). (81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> (88) Date of publication of the international search report: 15 June 2000 (15.06.00) | |
| (54) Title: USE OF BI-SPECIFIC ANTIBODIES FOR PRE-TARGETING DIAGNOSIS AND THERAPY | | | |
| (57) Abstract <p>The present invention relates to a bi-specific antibody or antibody fragment having at least one arm that specifically binds a targeted tissue and at least one other arm that specifically binds a targetable conjugate. The targetable conjugate comprises a carrier portion which comprises or bears at least one epitope recognizable by at least one arm of said bi-specific antibody or antibody fragment. The targetable conjugate further comprises one or more therapeutic or diagnostic agents or enzymes. The invention provides constructs and methods for producing the bi-specific antibodies or antibody fragments, as well as methods for using them.</p> | | | |

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INTERNATIONAL SEARCH REPORT

Intern. Application No
PCT/US 99/13879

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 A61K51/08 A61K47/48 C07K16/46 C12N15/62 C12N15/13
A01K67/027 //C12N5/10, C12N1/21

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Minimum documentation searched (classification system followed by classification symbols)

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Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

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C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|------------|---|-----------------------------------|
| X, P | KARACAY, H. ET AL: "Studies on a humanized anti-CEA x murine anti-(In- DTPA) bispecific antibody construct for radioimmunotherapy of CEA-positive tumors." PROCEEDINGS OF THE AMERICAN ASSOCIATION FOR CANCER RESEARCH ANNUAL MEETING, (MARCH, 1999) VOL. 40, PP. 644. MEETING INFO.: 90TH ANNUAL MEETING OF THE AMERICAN ASSOCIATION FOR CANCER RESEARCH PHILADELPHIA, PENNSYLVANIA, USA APRIL 10-14, 1999 AMERICAN, XP002120429 see abstract --- -/-- | 1,3, 12-17, 19-30, 32-34 |

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|--|---|-----------------------------------|
| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
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INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 99/13879

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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| X | <p>WO 96 04313 A (IMMUNOMEDICS INC) 15 February 1996</p> <p>see page 7, line 13-17 see page 37, line 14-17; example 4</p> | <p>1,3, 12-17, 19-30, 32-34</p> |
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INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 99/13879

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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| X | BARBET, J. ET AL: "Radioimmunotherapy of LS174T colon carcinoma in nude mice using an iodine-131-labeled bivalent hapten combined with an anti-CEAX anti-indium-DTPA bispecific antibody." TUMOR BIOLOGY, (SEPT., 1997) VOL. 18, NO. SUPPL. 2, PP. 31. MEETING INFO.: MEETING ON FROM BASIC CANCER RESEARCH TO CLINICAL APPLICATION HELD AT THE XXVTH ANNIVERSARY MEETING OF THE INTERNATIONAL SOCIETY FOR ONCODEVELOPMENTAL BIOLOGY AND MEDICINE MON, XP002120771 see abstract; claims 6,15; examples 5,8 --- | 1,3, 12-17, 19-30, 32-34 |
| X | US 5 591 828 A (BOSSLET KLAUS ET AL) 7 January 1997 see abstract; claims 6,15; example 5 ----- | 1,3, 12-17, 19-30, 32-34 |

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 99/ 13879

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
See annex
2. ☒ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
See annex
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

See annex

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

3 complete, 1, 12-17, 19-30, 32-34 partially

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

1. Claims: 3 complete, 1, 12-17, 19-30, 32-34 partially

Method of treating diseased tissues in a patient comprising:
A) administering a bi-specific antibody or antibody fragment having at least one arm that specifically binds a targeted tissue and at least one other arm that specifically binds a targetable conjugate

B) optionally administering a clearing composition and allowing to clear non-localized antibodies or antibody fragments from circulation.

C) administering a first targetable conjugate comprising a carrier portion that bears epitope recognizable by said one other arm of bi-specific antibody or antibody fragment and one or more conjugated radioactive isotope as therapeutic agent.

Pharmaceutical kits for performing the method.

2. Claims: 4, 5 complete, 1, 12-17, 19-30, 32-34 partially

Method of treating diseased tissues in a patient comprising:
A) administering a bi-specific antibody or antibody fragment having at least one arm that specifically binds a targeted tissue and at least one other arm that specifically binds a targetable conjugate

B) optionally administering a clearing composition and allowing to clear non-localized antibodies or antibody fragments from circulation.

C) administering a first targetable conjugate comprising a carrier portion that bears epitope recognizable by said one other arm of bi-specific antibody or antibody fragment and one or more conjugated boron compounds as therapeutic agent.

Pharmaceutical kits for performing the method.

3. Claims: 6 complete, 1, 12-17, 19-30, 32-34 partially

Method of treating diseased tissues in a patient comprising:
A) administering a bi-specific antibody or antibody fragment having at least one arm that specifically binds a targeted tissue and at least one other arm that specifically binds a targetable conjugate

B) optionally administering a clearing composition and allowing to clear non-localized antibodies or antibody fragments from circulation.

C) administering a first targetable conjugate comprising a carrier portion that bears epitope recognizable by said one other arm of bi-specific antibody or antibody fragment and one or more conjugated toxins as therapeutic agent.

Pharmaceutical kits for performing the method.

4. Claims: 7 complete, 1, 12-17, 19-30, 32-34 partially

Method of treating diseased tissues in a patient comprising:

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

- A) administering a bi-specific antibody or antibody fragment having at least one arm that specifically binds a targeted tissue and at least one other arm that specifically binds a targetable conjugate
 - B) optionally administering a clearing composition and allowing to clear non-localized antibodies or antibody fragments from circulation.
 - C) administering a first targetable conjugate comprising a carrier portion that bears epitope recognizable by said one other arm of bi-specific antibody or antibody fragment and one or more conjugated drug as therapeutic agent.
- Pharmaceutical kits for performing the method.

5. Claims: 18 complete, 1, 12-17, 19-30, 32-34 partially

- Method of treating diseased tissues in a patient comprising:
- A) administering a bi-specific antibody or antibody fragment having at least one arm that specifically binds a targeted tissue and at least one other arm that specifically binds a targetable conjugate
 - B) optionally administering a clearing composition and allowing to clear non-localized antibodies or antibody fragments from circulation.
 - C) administering a first targetable conjugate comprising a carrier portion that bears epitope recognizable by said one other arm of bi-specific antibody or antibody fragment and one or more conjugated hapten as therapeutic agent.
- Pharmaceutical kits for performing the method.

6. Claims: 9,11 complete, 1, 12-17, 19-30, 32-34 partially

- Method of identifying diseased tissues in a patient comprising:
- A) administering a bi-specific antibody or antibody fragment having at least one arm that specifically binds a targeted tissue and at least one other arm that specifically binds a targetable conjugate
 - B) optionally administering a clearing composition and allowing to clear non-localized antibodies or antibody fragments from circulation.
 - C) administering a first targetable conjugate comprising a carrier portion that bears epitope recognizable by said one other arm of bi-specific antibody or antibody fragment and one or more conjugated radioactive isotopes as diagnostic agent.
- Pharmaceutical kits for performing the method.

7. Claims: 10 complete, 1, 12-17, 19-30, 32-34 partially

- Method of identifying diseased tissues in a patient comprising:
- A) administering a bi-specific antibody or antibody fragment

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

having at least one arm that specifically binds a targeted tissue and at least one other arm that specifically binds a targetable conjugate

B) optionally administering a clearing composition and allowing to clear non-localized antibodies or antibody fragments from circulation.

C) administering a first targetable conjugate comprising a carrier portion that bears epitope recognizable by said one other arm of bi-specific antibody or antibody fragment and one or more conjugated MRI imaging agents as diagnostic agent.

Pharmaceutical kits for performing the method.

8. Claims: 1, 12-17, 19-30, 32-34 partially

Method of treating diseased tissues in a patient comprising:

A) administering a bi-specific antibody or antibody fragment having at least one arm that specifically binds a targeted tissue and at least one other arm that specifically binds a targetable conjugate

B) optionally administering a clearing composition and allowing to clear non-localized antibodies or antibody fragments from circulation.

C) administering a first targetable conjugate comprising a carrier portion that bears epitope recognizable by said one other arm of bi-specific antibody or antibody fragment and one or more conjugated enzymes

D) further administering a prodrug where said enzyme is capable of converting said prodrug to a drug at the target site.

Pharmaceutical kits for performing the method.

9. Claims: 1, 12-17, 19-30, 32-34 partially

Method of treating diseased tissues in a patient comprising:

A) administering a bi-specific antibody or antibody fragment having at least one arm that specifically binds a targeted tissue and at least one other arm that specifically binds a targetable conjugate

B) optionally administering a clearing composition and allowing to clear non-localized antibodies or antibody fragments from circulation.

C) administering a first targetable conjugate comprising a carrier portion that bears epitope recognizable by said one other arm of bi-specific antibody or antibody fragment and one or more conjugated enzymes

D) further administering a drug which is capable of being detoxified to form an intermediate of lower toxicity and said enzyme is capable of reconvertng the detoxified intermediate to a toxic form and therefore increasing toxicity of said drug at the target site.

Pharmaceutical kits for performing the method.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

10. Claims: 1,2,8 12-17, 19-34 partially

Method of treating diseased tissues in a patient comprising:
A) administering a bi-specific antibody or antibody fragment having at least one arm that specifically binds a targeted tissue and at least one other arm that specifically binds a targetable conjugate
B) optionally administering a clearing composition and allowing to clear non-localized antibodies or antibody fragments from circulation.
C) administering a first targetable conjugate comprising a carrier portion that bears epitope recognizable by said one other arm of bi-specific antibody or antibody fragment and one or more conjugated enzymes
D) further administering a prodrug which is activated through natural processes and subject to detoxification (conversion to a lower toxicity intermediate), where said enzyme is capable of reconvertng said detoxified intermediate to a toxic form and therefore increasing toxicity of said drug at the target site.
Pharmaceutical kits for performing the method.

11. Claims: 1,2,8 12-17, 19-34 partially

Method of treating diseased tissues in a patient comprising:
A) administering a bi-specific antibody or antibody fragment having at least one arm that specifically binds a targeted tissue and at least one other arm that specifically binds a targetable conjugate
B) optionally administering a clearing composition and allowing to clear non-localized antibodies or antibody fragments from circulation.
C) administering a first targetable conjugate comprising a carrier portion that bears epitope recognizable by said one other arm of bi-specific antibody or antibody fragment and one or more conjugated enzymes
D) further administering a second targetable conjugate which comprises a carrier portion that bears epitope recognizable by at least one other arm of bi-specific antibody or antibody fragment and a prodrug where said enzyme is capable of converting said prodrug to a drug at the target site.
Pharmaceutical kits for performing the method.

12. Claims: 35-50

A recombinant DNA construct comprising an expression cassette or a set of expression cassettes capable of producing in a host cell a bi-specific antibody or antibody fragment or bispecific fusion protein having at least one arm that specifically binds a targeted tissue and at least one other arm that specifically binds a targetable conjugate.
A method of preparing a bi-specific antibody or antibody

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

fragment or bispecific fusion protein having at least one arm that specifically binds a targeted tissue and at least one other arm that specifically binds a targetable conjugate.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box 3.

Although claims 1-29 are directed to a diagnostic method practised on the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

Although claims 1-29 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

Further defect(s) under Article 17(2)(a):

Continuation of Box 3.

Claims Nos.: 1, 3, 12-17, 19-30, 32-34

Present claims 1, 3, 12-17, 19-30, 32-34 relate to a method and the corresponding kit defined by reference (inter alia) to the following parameters: a clearing composition, a first targetable conjugate, conjugated therapeutic or diagnostic agents or enzymes, a prodrug, a drug, etcetera.

The claims cover all products having these characteristics or properties, whereas the application provides support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT for only a very limited number of such products. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Independent of the above reasoning, the claims also lack clarity (Article 6 PCT). Consequently, the search for the first invention has been carried out for those parts of the claims which appear to be clear, supported and disclosed, namely those parts relating to the products and methods mentioned in the description in examples and those specified in the claims and to the general idea underlying the present application.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Intern. nat. Application No

PCT/US 99/13879

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